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L1 317 VP22

=> s l1(s)carrier
L2 3 L1(S) CARRIER

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PROCESSING COMPLETED FOR L2
L3 3 DUP REM L2 (0 DUPLICATES REMOVED)

=> d ti so 1-3

L3 ANSWER 1 OF 3 CAPLUS COPYRIGHT 2002 ACS
TI Transiently immortalized cells for use in gene therapy
SO U.S. Pat. Appl. Publ., 22 pp., Cont.-in-part of U.S. Ser. No. 546,483.
CODEN: USXXCO

L3 ANSWER 2 OF 3 CAPLUS COPYRIGHT 2002 ACS
TI A genetic immunization adjuvant system based on BVP22-antigen fusion
SO Human Gene Therapy (2001), 12(10), 1353-1359
CODEN: HGTHE3; ISSN: 1043-0342

L3 ANSWER 3 OF 3 MEDLINE
TI A peptide carrier for the delivery of biologically active proteins into mammalian cells.
SO NATURE BIOTECHNOLOGY, (2001 Dec) 19 (12) 1173-6.
Journal code: 9604648. ISSN: 1087-0156.

=> d ibib ab 3

L3 ANSWER 3 OF 3 MEDLINE
ACCESSION NUMBER: 2001685487 MEDLINE
DOCUMENT NUMBER: 21588760 PubMed ID: 11731788
TITLE: A peptide carrier for the delivery of biologically active proteins into mammalian cells.
AUTHOR: Morris M C; Depollier J; Mery J; Heitz F; Divita G
CORPORATE SOURCE: Centre de Recherches de Biochimie Macromoleculaire,
UPR-1086 CNRS, 1919 Route de Mende, 34293 Montpellier,
Cedex 5, France.
SOURCE: NATURE BIOTECHNOLOGY, (2001 Dec) 19 (12)

1173-6.
Journal code: 9604648. ISSN: 1087-0156.
PUB. COUNTRY: United States
DOCUMENT TYPE: Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200202
ENTRY DATE: Entered STN: 20011204
Last Updated on STN: 20020215
Entered Medline: 20020214

AB The development of peptide drugs and therapeutic proteins is limited by the poor permeability and the selectivity of the cell membrane. There is a growing effort to circumvent these problems by designing strategies to deliver full-length proteins into a large number of cells. A series of small protein domains, termed protein transduction domains (PTDs), have been shown to cross biological membranes efficiently and independently of transporters or specific receptors, and to promote the delivery of peptides and proteins into cells. TAT protein from human immunodeficiency virus (HIV-1) is able to deliver biologically active proteins in vivo and has been shown to be of considerable interest for protein therapeutics. Similarly, the third alpha-helix of Antennapedia homeodomain, and **VP22** protein from herpes simplex virus promote the delivery of covalently linked peptides or proteins into cells. However, these PTD vectors display a certain number of limitations in that they all require crosslinking to the target peptide or protein. Moreover, protein transduction using PTD-TAT fusion protein systems may require denaturation of the protein before delivery to increase the accessibility of the TAT-PTD domain. This requirement introduces an additional delay between the time of delivery and intracellular activation of the protein. In this report, we propose a new strategy for protein delivery based on a short amphipathic peptide **carrier**, Pep-1. This peptide **carrier** is able to efficiently deliver a variety of peptides and proteins into several cell lines in a fully biologically active form, without the need for prior chemical covalent coupling or denaturation steps. In addition, this peptide **carrier** presents several advantages for protein therapy, including stability in physiological buffer, lack of toxicity, and lack of sensitivity to serum. Pep-1 technology should be extremely useful for targeting specific protein-protein interactions in living cells and for screening novel therapeutic proteins.

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=> s promoter
L2 339385 PROMOTER

=> s l1(s)l2
L3 315 L1(S) L2

=> s microinject?
L4 43088 MICROINJECT?

=> s l3 and l4
L5 2 L3 AND L4

=> dup rem l5
PROCESSING COMPLETED FOR L5
L6 2 DUP REM L5 (0 DUPLICATES REMOVED)

=> d ti so 1-2

L6 ANSWER 1 OF 2 CAPLUS COPYRIGHT 2002 ACS
TI Method of performing single shot double transgenesis (one manipulation)
involving **microinjection** of oocytes
SO PCT Int. Appl., 45 pp.
CODEN: PIXXD2

L6 ANSWER 2 OF 2 MEDLINE
TI FLP-mediated recombination in the vector mosquito, Aedes aegypti.
SO NUCLEIC ACIDS RESEARCH, (1991 Nov 11) 19 (21) 5895-900.
Journal code: 0411011. ISSN: 0305-1048.

=> d ibib ab 1

L6 ANSWER 1 OF 2 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 2000:133382 CAPLUS
DOCUMENT NUMBER: 132:176573
TITLE: Method of performing single shot double transgenesis
(one manipulation) involving **microinjection** of oocytes
INVENTOR(S): Perry, Anthony C. F.; Wakayama, Teruhiko
PATENT ASSIGNEE(S): USA
SOURCE: PCT Int. Appl., 45 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 2
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2000008924	A1	20000224	WO 1999-US18429	19990811

W: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
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AU 9955603 A1 20000306 AU 1999-55603 19990811
EP 1111991 A1 20010704 EP 1999-942164 19990811
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI, RO
BR 9913644 A 20011120 BR 1999-13644 19990811
JP 2002524054 T2 20020806 JP 2000-564438 19990811
PRIORITY APPLN. INFO.: US 1998-96078P P 19980811
US 1999-134251P P 19990513
WO 1999-US18429 W 19990811

AB The invention provides a method for generating transgenic animals and cells by the coinsertion of nucleic acid and a nucleus into an unfertilized oocyte cytoplasm using a **microinjection** pipet. The oocyte is immature and is arrested at the second metaphase (metII) of meiosis. The nucleus may be taken from an embryo or fetus or mammalian cell or somatic cell or gamete (oocyte or spermatozoon). The mammals may include primates, ovines, bovines, porcines, ursines, caprines, felines, canines, equines, and murines. Preferably, the coinsertion is by **microinjection** and more preferably by piezo-elec. actuated **microinjection**. Transgene (tg) expressing embryos are here produced following coinjection of unfertilized mouse oocytes with sperm heads and exogenous DNA encoding either a green fluorescent protein (GFP) or .beta.-galactosidase reporter. The authors show that sperm heads whose membranes have been disrupted by freezing or freeze-drying or detergent promote transgenesis with high efficiency. The detergent may be ionic or non-ionic. This allows transgene DNA to gain access to sub-nuclear elements, including the perinuclear matrix (in case of spermatozoa), the nuclear matrix, chromatin and genomic dna.,. The Cre-**lox** system was used to monitor transgenesis where loxP sites flank a tissue-specific **promoter**. This involves utility of site-specific recombination and uses site-specific recombinases, single-stranded DNA binding proteins, RNA binding proteins. reverse transcriptases, topoisomerases, endonucleases and recombinases that promote homologous recombination. The **microinjected** oocyte may be allowed to develop into differentiated cells or stem cells; into an embryo in vitro prior to transfer into a host surrogate mother; or it may be transferred directly into a host surrogate mother. **Microinjection** of tg DNA in absence of sperm nucleus suggests that sperm nuclear components sustain tg DNA in recombinogenic form. Embryonic development can occur to term, such that the offspring possess transgenic modifications that may alter their characteristics (phenotype) and are, in turn, transmitted to their offspring.

REFERENCE COUNT: 6 THERE ARE 6 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

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L1 4601 S LOX OR FRT
L2 339385 S PROMOTER
L3 315 S L1(S)L2
L4 43088 S MICROINJECT?
L5 2 S L3 AND L4
L6 2 DUP REM L5 (0 DUPLICATES REMOVED)

=> s l1 and l4
L7 21 L1 AND L4

=> dup rem l7
PROCESSING COMPLETED FOR L7
L8 10 DUP REM L7 (11 DUPLICATES REMOVED)

=> d ti so 1-10

L8 ANSWER 1 OF 10 CAPLUS COPYRIGHT 2002 ACS
TI Construction of recombinant vectors comprising a transcriptionally silent
element for conditional gene inactivation in mammalian cells
SO PCT Int. Appl., 58 pp.
CODEN: PIXXD2

L8 ANSWER 2 OF 10 MEDLINE DUPLICATE 1
TI Efficient FLP recombination in mouse ES cells and oocytes.
SO GENESIS, (2001 Sep) 31 (1) 6-10.
Journal code: 100931242. ISSN: 1526-954X.

L8 ANSWER 3 OF 10 CAPLUS COPYRIGHT 2002 ACS
TI Method of performing single shot double transgenesis (one manipulation)
involving **microinjection** of oocytes
SO PCT Int. Appl., 45 pp.
CODEN: PIXXD2

L8 ANSWER 4 OF 10 MEDLINE DUPLICATE 2
TI Formation of three-dimensional thyroid follicle-like structures by polarized **FRT** cells made communication competent by transfection and stable expression of the connexin-32 gene.
SO ENDOCRINOLOGY, (2000 Apr) 141 (4) 1403-13.
Journal code: 0375040. ISSN: 0013-7227.

L8 ANSWER 5 OF 10 MEDLINE DUPLICATE 3
TI Characterization of Cre-mediated cassette exchange after plasmid **microinjection** in fertilized mouse oocytes.
SO GENESIS, (2000 Aug) 27 (4) 153-8.
Journal code: 100931242. ISSN: 1526-954X.

L8 ANSWER 6 OF 10 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.DUPLICATE 4
TI Genomic plasticity and nuclear totipotency: Application to cloning and
transgenesis in domestic mammals.
Original Title: Plasticite du genome et totipotence nucleaire: Application
a la transgenese par clonage chez les mammiferes d'elevage..
SO Comptes Rendus de l'Academie d'Agriculture de France, (2000) Vol. 86, No.
4, pp. 83-97. print.
ISSN: 0989-6988.

L8 ANSWER 7 OF 10 MEDLINE DUPLICATE 5
TI Activation of beta1 integrin signaling stimulates tyrosine phosphorylation
of p190RhoGAP and membrane-protrusive activities at invadopodia.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1998 Jan 2) 273 (1) 9-12.
Journal code: 2985121R. ISSN: 0021-9258.

L8 ANSWER 8 OF 10 CAPLUS COPYRIGHT 2002 ACS
TI Production of somatic mosaicism in mammals using a gene that can be
activated or inactivated by regulatable somatic recombination
SO PCT Int. Appl., 66 pp.
CODEN: PIXXD2

L8 ANSWER 9 OF 10 MEDLINE DUPLICATE 6
TI FLP-mediated site-specific recombination in **microinjected** murine zygotes.
SO TRANSGENIC RESEARCH, (1996 Nov) 5 (6) 385-95.
Journal code: 9209120. ISSN: 0962-8819.

L8 ANSWER 10 OF 10 MEDLINE
TI FLP-mediated recombination in the vector mosquito, Aedes aegypti.
SO NUCLEIC ACIDS RESEARCH, (1991 Nov 11) 19 (21) 5895-900.
Journal code: 0411011. ISSN: 0305-1048.

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L2 339385 S PROMOTER
L3 315 S L1(S)L2
L4 43088 S MICROINJECT?
L5 2 S L3 AND L4
L6 2 DUP REM L5 (0 DUPLICATES REMOVED)
L7 21 S L1 AND L4
L8 10 DUP REM L7 (11 DUPLICATES REMOVED)

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Trends Pharmacol Sci 2000 Feb;21(2):45-8

Curr Opin Biotechnol 2000 Oct;11(5):461-6

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Curr Opin Mol Ther. 1999 Apr;1(2):226-43. Review.
PMID: 11715946 [PubMed - indexed for MEDLINE]

2 Schwartz JJ, Zhang S.
Peptide-mediated cellular delivery.
Curr Opin Mol Ther. 2000 Apr;2(2):162-7. Review.
PMID: 11249637 [PubMed - indexed for MEDLINE]

3: Sparrow JT, Edwards V V, Tung C, Logan MJ, Wadhwa MS, Duguid J, Smith LC.
Synthetic peptide-based DNA complexes for nonviral gene delivery.
Adv Drug Deliv Rev. 1998 Mar 2;30(1-3):115-131.
PMID: 10837606 [PubMed - as supplied by publisher]

4: Wadhwa MS, Collard WT, Adami RC, McKenzie DL, Rice KG.
Peptide-mediated gene delivery: influence of peptide structure on gene expression.
Bioconjug Chem. 1997 Jan-Feb;8(1):81-8.
PMID: 9026040 [PubMed - indexed for MEDLINE]

5: Brown MD, Schatzlein AG, Uchegbu IF.
Gene delivery with synthetic (non viral) carriers.
Int J Pharm. 2001 Oct 23;229(1-2):1-21. Review.
PMID: 11604253 [PubMed - indexed for MEDLINE

6: Gariepy J, Kawamura K.
Vectorial delivery of macromolecules into cells using peptide-based vehicles.
Trends Biotechnol. 2001 Jan;19(1):21-8. Review.
PMID: 11146099 [PubMed - indexed for MEDLINE]

7: Plank C, Zauner W, Wagner E.
Application of membrane-active peptides for drug and gene delivery across cellular membranes.
Adv Drug Deliv Rev. 1998 Oct 5;34(1):21-35.
PMID: 10837668 [PubMed - as supplied by publisher]

8: Lollo CP, Banaszczyk MG, Chiou HC.
Obstacles and advances in non-viral gene delivery.
Curr Opin Mol Ther. 2000 Apr;2(2):136-42. Review.
PMID: 11249633 [PubMed - indexed for MEDLINE]

9: Hawiger J.
Noninvasive intracellular delivery of functional peptides and proteins.
Curr Opin Chem Biol. 1999 Feb;3(1):89-94. Review.
PMID: 10021415 [PubMed - indexed for MEDLI

10: Lechardeur D, Lukacs GL.
Intracellular barriers to non-viral gene transfer.
Curr Gene Ther. 2002 May;2(2):183-94. Review.
PMID: 12109215 [PubMed - indexed for MEDLINE

11: Vives E, Brodin P, Lebleu B.
A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus.
J Biol Chem. 1997 Jun 20;272(25):16010-7.
PMID: 9188504 [PubMed - indexed for MEDLINE]

Peptide-mediated cellular delivery

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Current Opinion in Molecular Therapeutics (2000) 2(2):162-167
 ISSN 1464-8431

Peptide-mediated molecular therapeutic delivery systems have recently emerged as an alternative means to effectively substitute or augment present gene therapy technologies, eg, TAT, VP22, engineered peptides. These systems show great promise for the elimination of the main bottleneck to safe, efficient, targeted gene therapy delivery and are able to efficiently introduce DNA, antisense peptide nucleic acids, oligonucleotides, small molecules and proteins into cells both in vitro and in vivo. They are versatile and easily designed to incorporate a number of specific attributes required for efficient cargo delivery. A fundamentally new property of these moieties will allow the therapeutic intervention in the biochemistry of the target cell without the need to alter its genome.

Keywords Antennapedia, membrane translocation, molecular engineering, non-viral delivery, TAT, VP22

Introduction

Each cell in the body has a specific function and fate. Regulated gene expression determines the character of the cells in each organ, as well as their proper function. Residing within every somatic cell of the body is the complete genetic information required to make an entire organism. There are more than 100,000 genes in the human genome, but only a subset of genes are active in any cell at any given time. The correct regulation in temporal and spatial expression of the DNA blueprint is critical for proper cellular function. Disregulation of the genetic program by mutations in DNA usually results in pathology (eg, cardiovascular, cystic fibrosis, hemophilia and cancer).

The standard paradigm of somatic cell gene therapy is the ability to transfer genetic material into a non-germ diseased cell to induce a permanent cure. Gene therapy is especially appropriate in well characterized, monogenic diseases, for example, where the specific mutation is known and conventional pharmacological approaches are insufficient, ie, adenosine deaminase deficiency (ADA), hemophilia, cystic fibrosis, Duchenne muscular dystrophy, sickle cell and lung disease [1••,2-5]. Tailoring the treatment regime to specific molecular causes increases the therapeutic index of treatment and reduces its adverse side effects. This is the driving motivation behind gene therapy today.

The current problems and the ideal system

The lack of predictable safety and efficacy standards in somatic gene therapy systems, has brought the whole field to a crossroads. Replication-incompetent viruses, naked DNA injection and liposomal agents have been the predominant means of genetic transfer. To date, there has been little lasting impact in the typical practice of medicine conferred by these gene therapy technologies. The crux of today's gene therapy dilemma is still the same as it has always been: efficient, safe, targeted delivery and persistent gene expression [1••,6].

In light of the safety concerns and efficacy issues encountered with viral transduction [*http://www.med.upenn.edu/ihgt/jesse.html, http://www.med.upenn.edu/ihgt/findings.html*], peptide-based gene delivery agents are emerging as alternatives for safer *in vivo* delivery. The main attraction of these peptide systems is their versatility. Peptide-based delivery systems have the ability to deliver therapeutic proteins, bioactive peptides, small molecules and any size nucleic acids. The use of these agents allows the researcher to intervene at multiple levels in the cells genetics and biochemistry and is a fundamental new technology in the gene therapy field [7•,8•].

Peptide delivery agents are more like traditional pharmacological drugs than gene therapy vectors. With the past to guide us, a critical re-evaluation of the best characteristics for an ideal delivery system is in order. The desirable features may include the items displayed in Table 1. Peptide-based systems seem to have many of these features inherent in their design, or can be easily engineered to encompass them.

Table 1. Desirable features for a peptide-based delivery system.

Redundant safety features
Efficient transit into cells *in vivo*
Efficient, stable delivery of undegraded cargo into the nucleus
No size constraints on cargo
Aid in regulated, appropriate and sustained expression of any cargo gene
Amenable to molecular design and engineering
Easily and inexpensively produced at high purity
Efficient targeting
Non-immunogenic, antigenic or inflammatory
Adaptable to any cell type of cell or tissue
Non-toxic and biodegradable
Easy, non-invasive administration
Sufficient serum half-life

State-of-the-art f gene delivery syst ms

Viral delivery systems have been state-of-the-art for some time now. The most mature viral technologies are based upon engineered retroviruses, adenoviruses and adeno-associated viruses. Many adenoviral gene therapy protocols rely on the vascular system to deliver virus to the liver by intravenous (iv) injection, or transduction by inhalation. Severe adverse events can and sometimes do occur with direct injection of adenovirus. These adverse events are usually a result of direct immunogenicity and antigenicity of the virus or viral proteins. Almost half of the human population has circulating antibodies to adenovirus. Thus many humans are already 'primed' to resist adenovirus-based therapies. These problems and DNA cargo size constraints have hampered the effective use of these vectors in the clinic.

Liposome delivery systems are another mature technology for gene delivery. Liposomes are synthesized lipid micelle forms, which can incorporate DNA and proteins. Mixed micelles of positively charged and neutral lipids bind electrostatically with the backbone of the DNA to be delivered. This complex shields the cargo from degradation and helps transit across the lipid bilayers of cells. There is inherent competition in the assembly of micelles and the subsequent unpacking of their DNA cargo. This results from the affinity of the lipid head group for the DNA. This lipid interaction can hamper gene transit to the nucleus from the cytosol and therefore expression. Liposome-DNA preparations injected into the tail vein of rodents typically elicit a transient bolus of expression. In many experiments, the lipid is rapidly absorbed onto serum proteins in the liver. Lipid toxicity, lack of targeting and the lack of long-term expression are problems associated with this gene therapy technology. However, new preparations of lipids have addressed some of the toxicity and targeting issues, but long-term *in vivo* expression of cargo alleles remains transient.

Other non-viral delivery systems have been investigated as gene therapy delivery modalities. Delivery of genes and other molecules into cells can also be accomplished by micro-injection, electroporation, encapsulation within polymers, or other physical means. There have been other non-viral delivery alternatives designed and tested in the last few years. These mainly rely on organic polymer technologies. In such systems the polymer backbone is conjugated with biological peptides, such as RGD motifs, which facilitate the delivery [9-12]. It is still too early to assess their feasibility for clinical human gene therapy due to their relatively complex manipulation, lack of specificity, potential cellular toxicity and immunogenicity/antigenicity.

Most gene therapy agents that are in use today have only some of the attributes listed in Table 1. Not every feature on the list needs to be included for a locally effective treatment. For example, a small number of cells transduced by the gene therapy vector can elicit tumor regression. Transient expression of herpes simplex virus thymidine kinase (TK) in part of a tumor allows the killing of the lesion by the prodrug gancyclovir. The TK protein leaks out of dead and dying cells and is then taken up by neighboring tumor cells. This makes the untransduced cells equally susceptible to death because of gancyclovir's toxicity. The bystander effect can allow killing of the majority of the tumor by transducing a small portion with a prodrug activator gene.

The arly pr mis and the reality

In the early 1990s retroviral gene therapy protocols were used to treat ADA patients with limited success [13-15]. These trials typically relied on the ability of engineered retroviruses to efficiently enter and establish themselves in dividing cells *ex vivo*. Cells were transplanted back into the patient and the ADA gene product was expressed. The patients transiently expressed enough ADA protein for a time and then the expression decayed to non-therapeutic levels requiring the resumption of standard treatment regimes (the iv infusion of ADA protein). Complications encountered in this trial included the possibility of initiating cancer by insertional mutagenesis of the host cell with retroviruses and the lack of sufficient long-term expression of the transferred allele. Lack of expression of the transferred gene resulted from several problems including: inefficient transduction, regrafting, immune factors and transcriptional silencing of the viral promoters by methylation.

Peptide-mediated delivery

Many peptide- and protein-mediated delivery systems have been reported (Table 2) [16-20,21••,22••,23,24,25••,26,27,28•, 29-34]. However, several barriers to their use as delivery agents exist, such as degradation by cellular proteases and nucleic acids by nucleases *in vivo*, efficient uptake into cells in the absence of specific receptors and transporters and escape from the endosome/lysosomal compartment. These are surmountable obstacles; which are amenable to rapid, rational engineering design and analysis.

The transit of peptides and proteins across lipid bilayers is generally energetically unfavorable. However, there are several naturally occurring proteins, which can pass across the membrane unencumbered by the need for specific transporter schemes. Some examples of this type of protein are antennapedia (*Drosophila*), Tat (HIV) and VP22 (herpes). Several peptides derived from these (and other) protein sequences are available that can efficiently enter mammalian cells, transit to the nucleus or carry molecular cargoes into the cell (Table 2).

HIV Tat protein

The 86 amino acid HIV transcriptional activator protein Tat, can be synthesized in one cell and then released and transited into a neighboring cell [35,36]. A receptor-mediated event is not required for TAT to pass into a neighboring cell. HIV-1, as well as all other lentiviruses, encode a potent Tat. This protein binds to specific regions of the virus genome (tar) and recruits cellular factors to increase the efficient transcription of the proviral genome. Tat is an important virulence factor of HIV infection. The Tat protein has three domains: the cysteine-rich, basic and integrin-binding regions. This protein has many effects on various host cells including being pro-angiogenic in endothelial cells [37,38].

Tabl 2. P ptides used in DNA, oligonucleotides, peptide and protein delivery.

Name	Sequence (N→C)	Size (kDa)	Reference
Tat	YGRKKRRQRRR	11	[21••]
ANTp	RQIKIWFQNRRMKWKK	16	[22••]
W/R	RRWRRWWRRWWRRWRR	16	[23]
NLS*	TPPKKKRKVEDP	12	[16]
AlkCWK$_{18}$	CWKKKKKKKKKKKKKKKKK	20	[20]
DiCWK$_{18}$	K$_{18}$WCCWK$_{18}$	40	[20]
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	27	[24]
DipaLytic	GLFEALEELWEAK	13	[25••]
K$_{16}$RGD	K$_{16}$GGCRGDMFGCAK16RGD	46	[26]
P1	K$_{16}$GGCMFGCGG	25	[27]
P2	K$_{16}$OCRRARGDNPDDRCT	31	[27]
P3	KKWKMRRNQFWVKVQRbAK (B) bA	20	[28•]
P3a	VAYISRGGVSTYYSDTVKGRFTRQKYNKRA	30	[29]
P9.3	IGRIDPANGKTKYAPKFQDKATRSNYYGNSPS	32	[29]
Plae	PLAEIDGIELTY	12	[30]
Kplae	K$_{16}$GGPLAEIDGIELGA	30	[30]
cKplae	K$_{16}$GGPLAEIDGIELCA	30	[30]
MGP	GALFLGFLGGAAGSTMGAWSQPKSKRKV	27	[31,32]
HA2	WEAK (LAKA)$_2$LAKH (LAKA)$_2$LKAC	28	[33]
LARL4$_6$	(LARL)$_6$-NH-CH$_3$	24	[33]
Hel-11-7	KLLKLLLKLWLKLLKLLL	18	[33]
KK	(KKKK)$_2$GGC	NA	[17]
KWK	(KWKK)$_2$GCC	NA	[17]
RWR	(RWRR)$_2$GGC	NA	[17]
Loligomer	K9K2) (K4) (K8) GGKKKKK-NLS	NA	[34]

Single letter code for amino acids are used for all peptides. The sizes range from 11 to 46 kDa. There are no obvious consensus sequences or conserved motifs. Some have positively charged residues for DNA binding, and clusters of hydrophobic residues, especially those with aromatic side chains, which presumably further condense DNA and facilitate membrane translocation. This list is by no means complete.
*NLS = nuclear localization signal, only one NLS is listed here although there are many variations of NLS that have been described. Derossi *et al* [22••] described a table which listed many variations of ANTp and its derivatives. Plank *et al* [17] have listed many more branched peptides; only three examples are listed here.
NA = Not applicable

A Tat-derived peptide that can traffic across the membrane is the protein transduction domain (PTD); which is only 11 amino acids long. Protein translational fusions with this sequence (YGRKKRRQRRR) transit efficiently across the mammalian cell membrane *in vitro* and *in vivo* into the nucleus. 50 Proteins from 15 to 120 kDa have been tested and all enter target human and murine cells efficiently [21••,39]. Additionally, bioactive peptides with the PTD appended remain biologically active with the appended transit sequence. These chemically synthesized peptides can rapidly pass into cells, accumulating in the nucleus.

Translational fusions of the 120 kDa β-galactosidase with Tat have been synthesized and injected into the peritoneum of mice [21••]. The remarkable results of these studies indicates that the Tat-tagged protein is rapidly taken up by all the cells and tissues in the mouse, including the brain. The blood-brain barrier is usually only accessible to small, lipophilic molecules. β-Galactosidase crosslinked to 35 amino acid Tat peptide can also be efficiently internalized. In both of these studies, the cell-associated β-galactosidase protein was active in *in situ* and FACS analysis. Interestingly, the crosslinked β-galactosidase was reported not to accumulate in the brain of mice.

The discrepancy between the earlier report by Fawell *et al* [40] and the recent report by Schwarze *et al* may be a result of differing sample preparation times or different means of preparing the β-galactosidase protein. The sampling time reported by Fawell *et al* [40] was about 20 min, while a recent report quoted several hours [21••]. Schwarze *et al* describe a method for β-galactosidase preparation which may yield 'misfolded' protein [21••], but the 'molten globule' appearance of the β-galactosidase may aid in its transit. Schwarze *et al* used intraperitoneal delivery rather than tail vein injections [21••]. Further investigations into the mode of entry and biophysics of Tat chimeras in transit through biological membranes are warranted.

Unfortunately, there is no apparent specificity in the transfer of the β-galactosidase protein into any cell type. All cells seem to incorporate the β-galactosidase fusion [21••]. If this method were to be used to deliver the gancyclovir prodrug activator, herpes thymidine kinase protein into cells, then unwanted collateral cell death would result. The inclusion of a dominant targeting domain in the chimera could eliminate this type of damage.

Drosophila homeoprotein antennapedia

The *Drosophila* homeotic protein antennapedia (ANTp) is capable of transiting across the membranes of animals and acting at a distance [22••]. The biology of ANT has been reviewed by Dorn *et al* [41••]. Briefly, homeobox genes specify spatial units of body plan. There is a 180 bp homeobox sequence upstream of the genes which encode a

DNA binding site for the homeotic gene products. Homeotic genes are helix turn helix proteins. A derivative of 60 amino acids of ANTp can translocate across cell membranes and bind to the homeobox sequence. The 16 amino acids (RQIKIWFQNRRMKWKK) of the third helix of ANTp (part of the 60 amino acids) have been identified as the minimal unit which can cross the membrane bilayer and eventually accumulate in the nucleus. Interestingly, the presence of a neural cell adhesion molecule (NCAM) linked to α-2,8-polysialic acid increases the efficiency of transit 4-fold. The internalization of ANTp is energy-independent and functions efficiently at 4 °C, suggesting that the translocation mechanism may be based on amphiphilicity. Different derivatives of this peptide have been synthesized and tested for entry and are reviewed by Derossi *et al* [22]. The results suggest that the entry of ANTp relies on key tryptophan, phenylalanine and glutamine residues. Furthermore, the retroinverse and all D-amino acid forms also are translocated efficiently. Therefore, a specific helical structure is not a prerequisite for membrane translocation entry. A proposed means of ANTp entry is that there may be a two-step interaction. The first step is the electrostatic approximation of the positively charged residues to the negatively charged cell surface. Secondly, the hydrophobic residues may facilitate interaction and translocation with the membrane. The presence of α-2,8-polysialic acid increases the net charge of the cell surface, aiding this type of interaction. The association of the positively charged ANTp with the negatively charged sialic acid chains would yield a neutral sialic acid and result in a local pH change at the cell surface. This pH change might induce the insertion of the ANTp into the hydrophobic membrane, which would concentrate the ANTp on the cell surface and allow hydrophobic forces to take hold, permitting entry. Exit of the ANTp-sialic acid complex would occur as a result of transition to the more neutral pH of the cytosol. This buffering pH shift would result in the release of ANTp. Entry is not dependent on sialic acid and ANTp transduces many cell types that do not express this material. The passage of ANTp from the cytosol to the nucleus can occur by passive diffusion. However, the rate of nuclear entry and the abundance of basic residues infer the presence of a nuclear localization sequence (NLS). The successful transduction of DNA that is larger than oligonucleotides has not yet been reported, but many bioactive peptides have been shown to traffic into cells when the ANTp sequence is added to it (Table 2).

Herpes virus VP22

Herpes virus VP22 tegument is another unusual protein molecule, which can efficiently traffic across cell membranes [42••,43,44•,45-47]. It is so efficient that expression in a small population of cells allows VP22 protein to traffic intercellularly to all cells of the culture. This protein concentrates in the nucleus and binds to chromatin. VP22 appears to traffic through the membrane via non-classical endocytosis and can enter cells regardless of GAP junctions and physical contacts [45]. Transit of VP22 is susceptible to actin cytoskeleton disruption. This protein colocalizes to cellular microtubules and causes them to reorganize into bundles. VP22 is also the target of phosphorylation. During viral infection, the VP22 that accumulates in the nucleus is phosphorylated, while that found in the cytoplasm is not. This phosphorylation is likely a result of casein kinase II activity, as VP22 has four amino proximal serine residues in consensus sites. The last 34 amino acids are critical for transit. It is important to note that only carboxyl-terminal translational fusion proteins can be carried into cells. The biological activities of several proteins (p53, GFP, thymidine kinase and others) have been investigated as translational fusions with VP22 [44•,45,46]. The VP22 cloning and expression system is now commercially available to investigators (Invitrogen Inc, *http://www.invitrogen.com*).

Approximately half of all cancers have diminished p53 activity [48,49]. Supplementing a 'good' copy of the gene product to these cells could induce them to undergo apoptosis and kill the tumor. The VP22-p53 chimerical protein retained its ability to spread to between cells and its proapoptotic activity, and had a widespread cytotoxic effect in p53 negative human osteosarcoma cells [44•]. Normal and supernormal levels of p53 are not usually harmful to normal cells. Unwanted side effects should be minimal with this type of cancer treatment.

HSV-TK linked in frame with VP22, was transported between the cells of a neuroblastoma culture and had TK activity. These cells are GAP junction negative [46]. Treatment of tissue culture cells transfected with TK protein alone, or VP22-TK protein with gancyclovir demonstrated that only the VP22-TK-treated cells died. Tumors established with a neuroblastoma cell line expressing VP22-TK regressed, while those expressing TK alone did not [46]. These results suggest that this novel method of cancer therapy holds promise in the clinic.

VP22-GFP (DNA expression plasmid) was transfected into COS7 cells. These cells were then cocultured with target C2C12 myotubes. The C2C12 cells were permissive to efficient VP22-GFP entry [46]. Terminally differentiated skeletal muscle cells were also transduced with a VP22-GFP protein, which suggests that mitosis is not a requirement for efficient entry [46].

Unfortunately, immunogenicity may be a problem with VP22 chimeras. VP22-reactive T-cells may have a role to play in the control of recurrent HSV infection. The antiviral functions of infiltrating CD4-bearing T-cells may include cytotoxicity, inhibition of viral growth, lymphokine secretion and support of humoral and CD8 responses. Viral VP22 and dUTPase are known to be T-cell antigens [50]. Analysis of the clonal reactive attributes of T-cells recovered from herpetic lesions from several patients proved this point. Some VP22 CD4 T-cells exhibited cytotoxic activity against HSV infected cells. VP22 and dUTPase are now being evaluated as possible candidates for protective vaccination epitopes. While many of the attributes of VP22 are laudable, the fact that there might be a vigorous immune reaction to the chimera *in vivo* should be considered carefully when using this method in human gene therapy.

Oth r peptide-mediat d gen delivery systems

Peptide-mediated delivery systems including transportan, MPG, SCWKn, (LARL)n, HA2, RGD, NLS, loligomer and others (Table 2) have been reported. Transportan, is a chimerical peptide derived from the first 12 residues of

galanin connected by an ε-amino of a lysine with a 14 residue mastoparan. Mastoparan can effectively deliver oligonucleotides into cells in culture and animals to block cell signaling activities [24]. The MPG peptide is also a chimera; it is comprised of part of the HIV gp41 protein and the NLS of SV40 large T-antigen. MPG can act a potent gene delivery agent [31,32]. The cell adhesion motif RGD linked with oligolysine has been reported to deliver genes into cells [26]. Alterations of the region bounding the RGD motif also allow cell type-specific targeting.

Conclusions

Peptide- and protein-mediated gene delivery systems are currently not the state-of-the-art choice for delivery. However, these systems are extremely versatile and amenable to rational design and modification. Peptides can also include many unique features like cell tropism and flexible cargo delivery. Problems of antigenicity inherent in peptide system design can be eliminated or minimized. ANTp has been shown to be only weakly immunogenic in mice. *In vitro* protein/peptide evolution and *in vivo* phage-display technologies [51••,52,53, *http://www.phylos.com*] allows the discovery of additional peptides/proteins that are more efficient, safe and specific for targeted delivery. We expect that out of this emerging field, peptide systems will play an increasing role in targeted molecular therapeutics and gene therapy.

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Intracellular Barriers to Non-Viral Gene Transfer

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Abstract: Non-viral vector mediated gene transfer, compared to viral vector mediated one, is a promising tool for the safe delivery of therapeutic DNA in genetic and acquired human diseases. Although the lack of specific immune response favor the clinical application of non-viral vectors, comprising of an expression cassette complexed to cationic liposome or cationic polymer, the limited efficacy and short duration of transgene expression impose major hurdles in the widespread application of non-viral gene therapy. The trafficking of transgene, complexed with chemical vectors, has been the subject of intensive investigations to improve our understanding of cellular and extracellular barriers impeding gene delivery. Here, we review those physical and metabolic impediments that account, at least in part, for the inefficient translocation of transgene into the nucleus of target cells. Following the internalization of the DNA-polycation complex by endocytosis, a large fraction is targeted to the lysosomal compartment by default. Since the cytosolic release of heterelogous DNA is a prerequisite for nuclear translocation, entrapment and degradation of plasmid DNA in endo-lysosomes constitute a major impediment to efficient gene transfer. Only a small fraction of internalized plasmid DNA penetrates the cytoplasm. Plasmid DNA encounters the diffusional and metabolic barriers of the cytoplasm, further decreasing the number of intact plasmid molecules reaching the nuclear pore complex (NPC), the gateway of nucleosol. Nuclear translocation of DNA requires either the disassembly of the nuclear envelope or active nuclear transport via the NPC. Comparison of viral and plasmid DNA cellular trafficking should reveal strategies that viruses have developed to overcome those cellular barriers that impede non-viral DNA delivery in gene therapy attempts.

INTRODUCTION

Both toxicological and ethical considerations favor the utilization of synthetic vectors over viral delivery systems to alleviate the phenotypic manifestations of genetic or acquired human diseases. Despite recent improvements of synthetic vectors, their application is still hampered by the low transduction efficiency of target cells *in vivo*.

One of the most widely used synthetic DNA delivery systems comprises of an expression cassette, inserted into a plasmid and complexed with cationic polymer (polyplex), cationic lipid (lipoplex) or a mixture of these (lipopolyplex). The positively charged DNA complex is taken up from the extracellular compartment by endocytosis and transferred into the nucleus of the target cell, an absolute prerequisite for successful gene expression. Although the accessibility and specific characteristics of the target organ may impose additional impediments to systemic gene delivery, the phospholipid membranes delineating the intracellular compartments, including the nucleosol, constitute major obstacles to the delivery of therapeutic genes.

Investigations of the cellular itinerary of DNA vectorized by synthetic molecules have provided insight into the nature of potential barriers to gene transfer. Once internalized, DNA has to escape from serial barriers, represented by endo-lysosomal entrapment, cytosolic sequestration, and nuclear exclusion. Besides these physical barriers, the DNA is also subjected to metabolic degradation, further compromising the efficiency of gene transfer.

In this review we provide an overview of the intracellular obstacles impeding the nuclear accumulation of plasmid DNA. Strategies developed by viruses to bypass these cellular barriers to ensure the nuclear delivery of the viral genome will be summarized briefly.

I) INTERNALIZATION, ENTRAPMENT AND DEGRADATION OF DNA IN THE ENDO-LYSOSOMAL COMPARTMENT

Morphological studies at light and electron microscopic levels suggest that following the electrostatic adsorption of positively charged lipoplex and polyplex on negatively charged plasma membrane, clathrin-dependent endocytosis is predominantly responsible for the cellular uptake of the complex [Clark and Hersh, 1999; Meyer *et al.*, 1997]. Direct fusion with the cell membrane and/or fluid phase endocytosis may also contribute to the cellular uptake of the complex. The size as well as the composition of the complex might determine the mechanism of internalization. Large lipoplex (up to 500 nm) enters the cell by receptor- and clathrin-independent endocytosis while the smaller complex (<200 nm) could be internalized via coated pits through a non-specific clathrin-dependent process [Simoes *et al*, 1999]. Cell surface properties and endocytic activity of the specific cell type also influence the contribution of

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various internalization pathways in the uptake of lipoplex [Fasbender *et al.*, 1997b; Jiang *et al.*, 1998; Matsui *et al.*, 1997; Remy-Kristensen *et al.*, 2001; Simoes *et al.*, 1999].

Endocytosis

Endocytosis comprises of those cellular events that lead to the internalization of specialized regions of the plasma membrane as well as small volumes of extracellular fluid [Mukherjee *et al.*, 1997]. The best known form of endocytosis is initiated by clathrin coated pits formation and involves the localized accumulation of clathrin chains with the AP-2 adaptor complexes. Association of the dynamin GTPase ensures the fission of the clathrin coated vesicles, followed by rapid uncoating and fusion with early endosomes [Takei and Haucke, 2001]. Others forms of endocytosis include caveolae-mediated and clathrin-independent internalization [Nichols and Lippincott-Schwartz, 2001]. Finally, some cell types are capable of internalizing extracellular fluid via macropinocytosis and large particules via phagocytosis [Apodaca, 2001]. In non-polarized cells, internalized material is first targeted to tubulovesicular sorting endosomes, characterized by the association of Rab5 GTPase and early endosomal antigen1 (EEA1) [Christoforidis *et al.*, 1999]. While the vesicular compartment of early endosomes, enriched in soluble cargo, is targeted to late endosomes and subsequently to lysosomes, the membrane-rich tubular portion delivers its cargo preferentially to recycling endosome, confined to the pericentriolar region of the cell. Membrane proteins are delivered back to the cell surface from the recycling endosomes [Sheff *et al.*, 1999]. Endosomes can associate with both actin and microtubule (MT) based motor proteins and the intracellular trafficking and processing of endocytic vesicles are regulated by the cytoskeleton network [Apodaca, 2001].

Internalization of Lipoplex

Electron-dense cationic lipopolylysine containing liposomes have been visualized in endosomal compartment within one hour of administration by electron microscopy [Zhou and Huang, 1994]. Similar studies showed the localization of gold-labeled DNA complexed with DMRIE/DOPE (1,2-dimyristyloxypropyl-3-dimethylhydroxy-ethyl-ammonium bromide/ dioleoylphosphatidylethanol-amine) lipids in cytoplasmic vesicles, representing, most likely, endosomes [Zabner *et al.*, 1995]. Very little is known about the physico-chemical events and molecular interactions determining the fate of lipoplex in early endosomes. Endocytosed lipoplex could be routed for recycling to the extracellular compartment, targeted to lysosomes via late endosomes, and released into the cytoplasm "Fig. (1)". Regardless of the subsequent trafficking pathway of

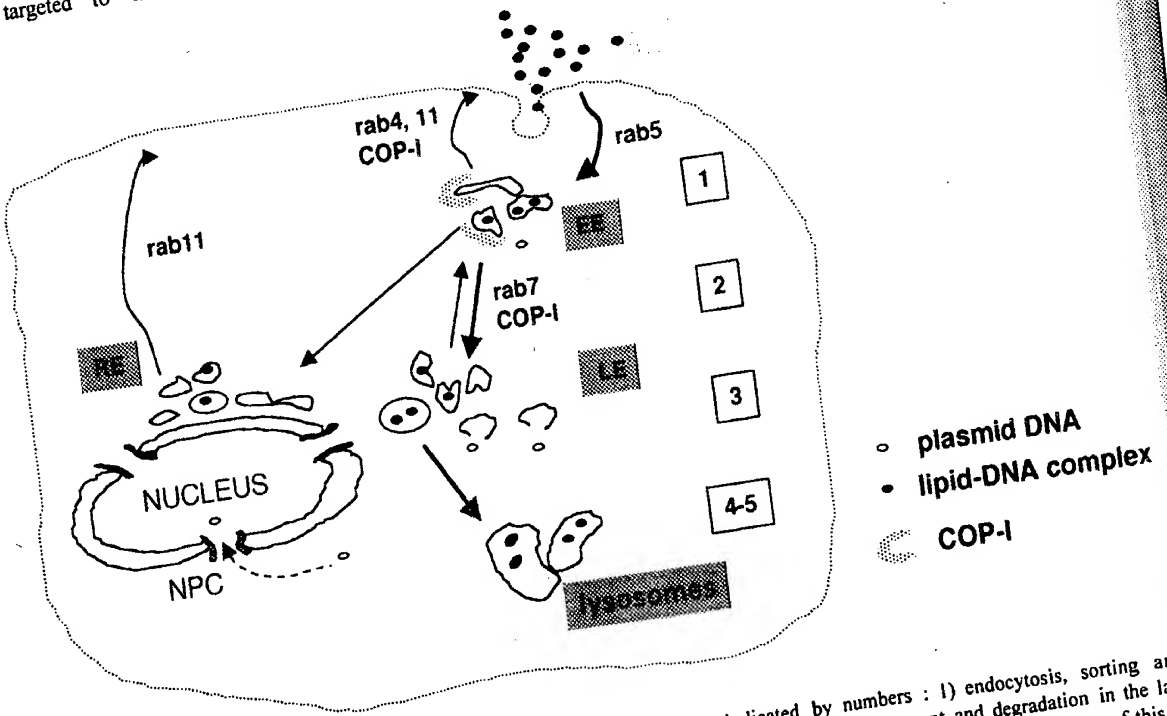

Fig. (1). The intracellular trafficking of plasmid DNA. Critical steps are indicated by numbers : 1) endocytosis, sorting and recycling via vesicular compartments comprising the early (EE) and sorting endosomes, 2) entrapment and degradation in the late endosomes (LE) and lysosomes, destabilization of the endo-lysosomal membrane and release in the cytosol (the location of this is not known), 4) diffusion toward the nuclear pore complex (NPC) and hydrolysis in the cytoplasm mediated by DNase and 5) nuclear translocation.

internalized plasmid DNA, penetration of nucleic acid into the cytoplasm seems to be a prerequisite for nuclear delivery.

The release of nucleic acids from the luminal compartment is thought to be a consequence of the disruption of the limiting membrane of endoso-lysosomes [El Ouahabi *et al.*, 1997; Wattiaux *et al.*, 2000; Xu and Szoka, 1996; Zelphati and Szoka, 1996a]. Disruption of endo-lysosmal membrane would occur through the interaction of the cationic lipid of the lipoplex by transbilayer flip-flop of anionic lipids from the external layer of the membrane [Zelphati and Szoka, 1996a; Zelphati and Szoka, 1996b]. According to the model, these interactions would eventually induce the release of the DNA from the lipoplex and the destabilization of the membrane, leading to the penetration of naked plasmid DNA into the cytoplasm [Mui *et al.*, 2000]. Other studies have demonstrated that cytoplasmic release of internalized lipoplex involves; a) charge neutralization of the cationic complexing agent with anionic macromolecules such as anionic lipids and proteoglycans, b) cationic lipid mediated fusion and c) membrane destabilization by pH-sensitive lipids [Clark and Hersh, 1999; Meyer *et al.*, 1997; Wrobel and Collins, 1995]. Mixing the neutral lipid DOPE with cationic lipid has been shown to increase the efficiency of gene transfer. This fusogenic lipid promotes the fusion of lipid/DNA particles with the endosomal membrane, facilitating membrane disruption and increasing the amount of plasmid molecules released into the cytoplasm [Farhood *et al.*, 1995, Fasbender, 1997 #40; Hafez *et al.*, 2001]. The efficacy of the cationic polyethylenimine (PEI) has been related to its extensive buffering capacity, provoking the swelling and disruption of endosomes [Klemm, 1998].

Regardless of the precise mechanism of membrane disruption, only a small fraction of internalized lipoplex reaches the cytoplasm, while a larger portion is trapped and eventually degraded in endo-lysosomes [El Ouahabi *et al.*, 1997; Hasegawa *et al.*, 2001; Plank *et al.*, 1994; Wattiaux *et al.*, 2000; Zabner *et al.*, 1995]. Accordingly, a variety of fusogenic agents, incorporated in lipopolyplex, could destabilize the endosomal membrane and enhance the cytoplasmic release and expression of reporter genes [Uherek *et al.*, 1998]. Adenoviral particles [Diebold *et al.*, 1999; Wagner *et al.*, 1992a; Wagner *et al.*, 1992b], peptides of viral origin (e.g. the haemagglutinin HA2 peptide from influenza virus [Wagner *et al.*, 1992a]), and subunits of toxins have been successfully utilized as fusogens. As a complementary approach, the transfection efficiency could be potentiated by dissipating the acidic pH endo-lysosomes and inhibiting the degradation of plasmid DNA using non-specific lysomotropic agents, such as chloroquine, polyvinylpyrolidone or sucrose [Ciftci and Levy, 2001; Harbottle *et al.*, 1998; Luthman and Magnusson, 1983; Niidome *et al.*, 1997]. Alternatively, lysosomal nuclease activity could be blocked by DMI-2, specific inhibitor of DNase I [Ross *et al.*, 1998]. Disrupting the microtubule network required for endo-lysosome fusion modestly enhanced transgene expression, by delaying the delivery to the lysosomal compartment [Chowdhury *et al.*, 1996; Hasegawa *et al.*, 2001]. These observations favor the notion that a significant fraction of internalized plasmid DNA is targeted to and degraded in lysosomes.

II) SEQUESTRATION AND DEGRADATION OF PLASMID DNA IN THE CYTOPLASM

Considering that plasmid DNA in the polycation complex is not accessible to the transcriptional machinery neither *in vitro* nor following microinjection into the nucleus [Zabner *et al.*, 1995], it was inferred that dissociation of the complex must precede its nuclear uptake via a nuclear pore complex (NPC). Following the endosomal escape, dissociation of polycation-plasmid DNA occurs in the cytosol, as demonstrated by the T7 polymerase transfection system, permitting the cytosolic transcription of reporter gene controlled by the T7 promoter [Brisson and Huang, 1999; Fasbender *et al.*, 1997a; Gao and Huang, 1993; Subramanian *et al.*, 1999]. This conclusion suggested that naked plasmid DNA is transiently exposed to the cytoplasm during its cellular itinerary and raised the possibility that the physico-chemical and biological properties of the cytosol may further impede the gene transfer efficiency of synthetic vectors.

The Cytoplasm

The cytoplasm is composed of a network of microfilament and microtubule systems, and a variety of subcellular organelles bathing in the cytosol. The cytoskeleton is responsible for the mechanical resistance of the cell, as well as the cytoplasmic transport of organelles and large complexes as ribonucleotides particules [Luby-Phelps, 2000]. Three classes of filaments constitute the mesh-like structure of the cytoplasm; actin filaments, microtubules and intermediate filaments. Synergic interactions between the different filaments and regulation by multiple actin-, microtubule-binding proteins, kinases and phosphatases are involved in the cohesion and maintenance of the structure of the cell [Fuchs and Yang, 1999].

The cytoskeleton is embedded in the fluid phase cytoplasm that contains macromolecules and small organic and inorganic solutes. Determination of rotational correlation time of fluorescent probe by time-resolved anisotropy [Fushimi and Verkman, 1991] and ratio-imaging of a viscosity-sensitive fluorescent probe [Luby-Phelps *et al.*, 1993] have revealed that the solvent viscosity of the cytoplasm is comparable to that of water. The mesh-like structure of the cytoskeleton, the presence of organelles and the high protein concentration (up to 100 mg/ml) impose an intensive molecular crowding of the cytoplasm which limits the diffusion of large sized macromolecules [Luby-Phelps, 2000]. Fluorescence recovery after photobleaching (FRAP) measurements of size-fractionated fluorescein-labeled ficolls and dextrans, delivered by microinjection, have demonstrated that the translational mobility of macromolecule smaller than 500-750 kDa is only 3-4 fold slower than in water, but is markedly impeded for larger molecules [Seksek *et al.*, 1997]. However, the intracytoplasmic diffusion of proteins is usually slower than inert particles (e.g. : ficolls or dextrans) due to binding interactions with intracellular components. Consequently, the diffusion coefficient of large size solutes is not correlated to their size or radius. Cytoplasmic vesicles or microinjected beads diffuse in the cytoplasm 500 to 1000 times slower

than in aqueous solution [Burke *et al.*, 1997; Luby-Phelps, 2000; Steiner *et al.*, 1997]. This restricted mobility of intracellular vesicles necessitates their active, vectorial transport, mediating movements along the cytoskeletal network [Klopfenstein *et al.*, 2000; Rogers and Gelfand, 2000].

Diffusional Properties of Plasmid DNA in the Cytoplasm

To study the dynamic properties of naked DNA in the cytoplasm, microinjection technique was utilized to deliver plasmid DNA directly into the cell. Dowty *et al.* (1995) were the first to discover that the diffusional mobility of plasmid DNA was negligible in the cytoplasm of microinjected myotubes. Plasmid DNA remained predominantly at the site of microinjection during incubation at 37 °C, suggesting that the cytoplasm sieving could represent an impediment to gene transfer [Dowty *et al.*, 1995].

Lateral diffusion of macromolecules with comparable size to expression cassettes (corresponding to 2-10 MDa molecular mass) suggests that the mobility of plasmid DNA is severely impeded in the cytoplasm. The limited cytoplasmic diffusion of plasmids or double stranded DNA fragments larger than 1 kb could be visualized by injecting fluorescein isothiocyanate (FITC)-conjugated DNA into the cytosol of HeLa cells. In contrast to oligonucleotides (20 bp) or DNA fragments smaller than 250 bp, which were able to diffuse into the nucleus, nucleic acids larger than 250 bp were excluded from the nuclei following a 45 min incubation at 37 °C "Fig. (**2a**)" [Leonetti *et al.*, 1991; Lukacs *et al.*, 2000].

To quantitatively determine the diffusion mobility of FITC-conjugated plasmid DNA (3-6 kb) and DNA fragments (20 bp-2 kb) in living cells, spot photobleaching technique was utilized. Following the microinjection of FITC-labeled DNA into the cytoplasm or the nucleus, a 0.4 μm diameter spot was bleached with high intensity laser beam and the time course of fluorescence-recovery was recorded [Lukacs *et al.*, 2000]. Nucleic acids larger than 2 kb have a very limited mobility and are virtually immobile during the course of the measurements (few minutes) in the cytoplasm of HeLa cells. Diffusion of larger DNA fragments became remarkably slower in the cytoplasm. Diffusion of 250 bp and 2000 bp fragments is 17 and >100 times slower, respectively, than diffusion in water [Lukacs *et al.*, 2000]. The restricted mobility of plasmid, DNA relatively to comparable size of dextran, could be explained by molecular crowding, immobile cytoplasmic obstacles or association of the nucleic acids with cytosolic DNA binding proteins. Since microinjected oligonucleotides exhibit homogenous distribution in the cytoplasm, the size more than interactions of the plasmid DNA with cytosolic proteins is thought to be responsible for the poor diffusional characterisitics of plasmid size DNA [Leonetti *et al.*, 1991].

Consistent with the notion that lateral diffusion may limit nuclear entry, microinjection of plasmid DNA into the proximity of the nucleus or decreasing the size of the expression cassette led to significant enhancement of the transfection efficiency [Darquet *et al.*, 1999; Dowty *et al.*, 1995]. Since the mobility of DNA is inversely proportional with the size of the polycation-DNA complex, it is reasonable to assume that the faster mobility of condensed DNA could account, at least in part, for enhanced transfection efficiency of the PEI-complexed plasmid DNA [Pollard *et al.*, 1998]. Intriguingly, double-stranded DNA fragments of 1kb size could enter the majority of nuclei in digitonin permeabilized cells but failed to reach the nucleus after microinjection in the cytoplasm [Hagstrom *et al.*, 1997] Fig. (**2**). These discordant results might be explained by the disassembly of the cytoskeletal network during permeabilization [Cook *et al.*, 1983] and reinforce the hypothesis that the cytoplasm constitutes a diffusional barrier to gene transfer.

Metabolic Instability of Plasmid DNA in the Cytoplasm

Microinjected DNA disappears in a time dependent manner from the cytosolic compartment, monitored by fluorescent in situ hybridization (FISH) [Lechardeur *et al.*, 1999]. This observation raised the possibility that metabolic instability of naked DNA may contribute to the low efficacy of gene transfer [Lechardeur *et al.*, 1999; Mirzayans *et al.*, 1992; Neves *et al.*, 1999]. Similar conclusion was reached by Pollard *et al.* (2001) by monitoring the presence of expression cassette by the polymerase chain reaction (PCR) technique in microinjected cells [Pollard *et al.*, 2001]. Quantitative assessment of decay kinetics of the FISH signal of microinjected plasmid DNA by single-cell video image analysis revealed that 50 % of the DNA is eliminated in 1-2 hours from HeLa and COS-1 cells [Lechardeur *et al.*, 1999] and in ≈ 4 hours from C2C12 cells and myotubes (F. Pampinella *et al.* unpublished observation). The fast turnover rate of microinjected DNA was independent of the copy number (1000-10,000 plasmid/cell) and the conformation (linearized vs. supercoiled , single- vs. double-stranded) of the plasmid delivered. Cytosolic elimination of plasmid DNA could not be attributed to cell division, since comparable degradation was observed in cell cycle arrested cells. Generation and subsequent elimination of free 3'-OH DNA ends, detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay, reflects the fragmentation of microinjected DNA *in situ* [Lechardeur *et al.*, 1999].

In vitro studies have demonstrated that complex formation can dramatically increase the nuclease resistance of plasmid DNA [Cappaccioli *et al.*, 1993; Chiou *et al.*, 1994; Thierry *et al.*, 1997]. Consistent with the diminished nuclease susceptibility of complexed DNA, encapsulation of microinjected plasmids into stabilized lipid particle delayed the degradation of DNA more than three-fold [Lechardeur *et al.*, 1999]. These results provide a plausible explanation for the increased efficacy of microinjected plasmid DNA, complexed by PEI [Pollard *et al.*, 1998]. It is conceivable that faster diffusional mobility as well as augmented nuclease resistance account for the enhanced nuclear targeting of the PEI-condensed plasmid DNA. Furthermore, these recent findings also suggest that the rapid degradation of plasmid DNA in the cytosol imposes an additional impediment to the nuclear translocation of DNA.

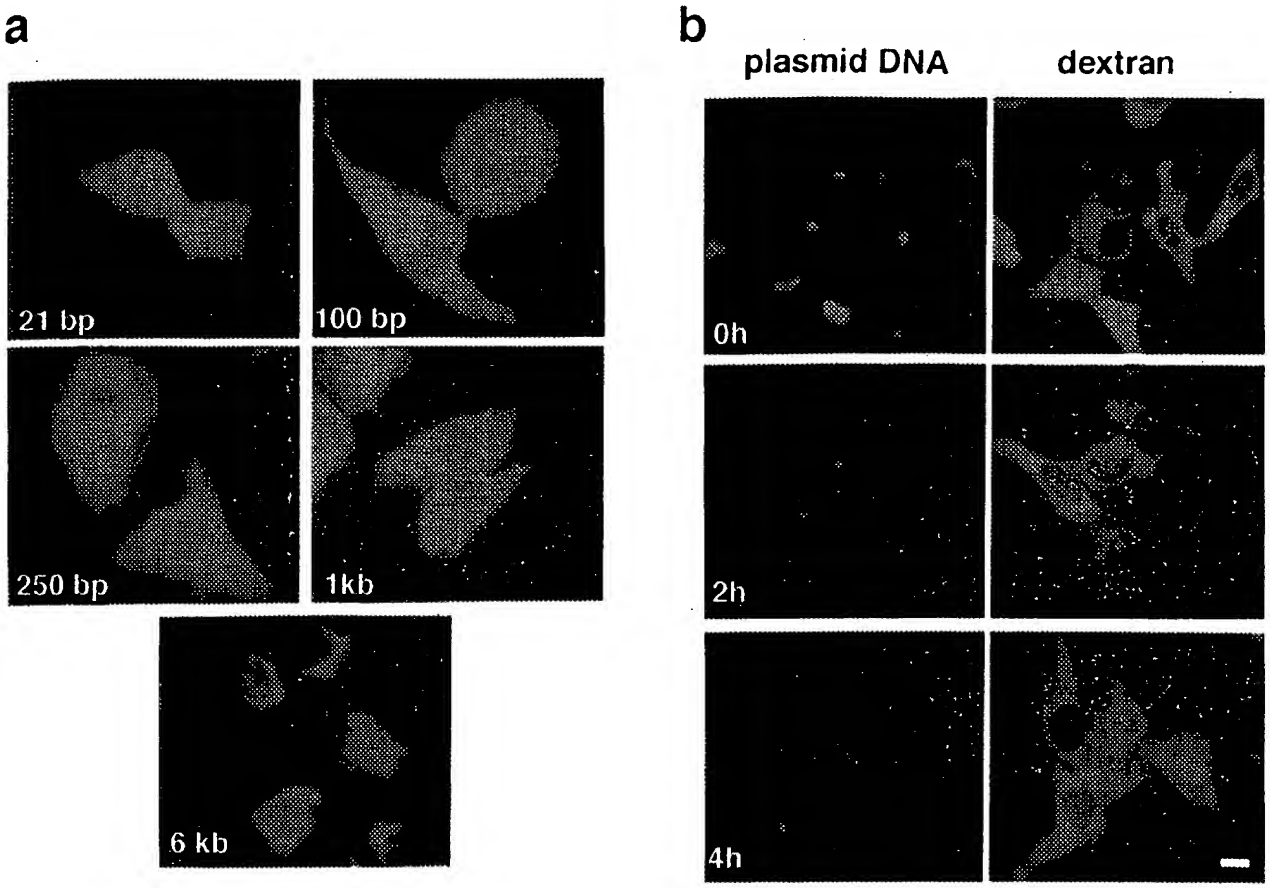

Fig. (2). The cytoplasm as a barrier to gene transfer. (A) Diffusion of microinjected fluorescein-labelled DNA fragments and plasmid DNA in the cytoplasm. Double-stranded circular plasmid DNA (3kb and 6kb) and DNA fragments (20, 100, 250 and 1 kb) were covalently labeled with fluorescein and microinjected into the cytoplasm of adherent cells as described [Lukacs *et al.*, 2000]. Following microinjection, cells were either fixed or incubated for 45 min at 37 °C and the distribution of DNA was visualized by fluorescence microscopy. (B) Degradation of plasmid DNA in the cytoplasm of microinjected cells. HeLa cells were co-injected fluorescein-labeled double-stranded pGL2 plasmid (0.1 μg/ml) (left panel) and TRITC-dextran (MW : 70 kDa) (right panel). Cells were incubated under tissue culture conditions for the indicated time and fluorescence micrographs were taken of the same cell population. Bar represents 10 μm.

Numerous cellular endo- and exonucleases have been described, but their function and subcellular localization are poorly understood [Peitsch *et al.*, 1994; Torriglia *et al.*, 1998; Vanderbilt *et al.*, 1982; Walker *et al.*, 1999]. Activation of some of these nucleases occurs during the initiation of programmed cell death (apoptosis) and plays a central role in the condensation and cleavage of chromosomal DNA [Torriglia *et al.*, 1995]. Some of the nucleases, like DNase I and DNase II, are thought to be released from intracellular organelles into the cytoplasm and subsequently translocated into the nucleus in apoptotic cells [Barry and Eastman, 1993; Polzar *et al.*, 1993; Wyllie *et al.*, 1980]. Others are constitutively expressed as inactive enzyme, like the Caspase-Activated DNase (CAD) or the L-Dnase II are activated through proteolytic cleavage in the nucleus [Lechardeur *et al.*, 2000] or translocated upon activation [Enari *et al.*, 1998; Sakahira *et al.*, 1998]. Since the apoptotic propensity of microinjected cells was not enhanced, it is unlikely that DNases invoked in chromosomal DNA degradation are responsible for the disappearance of microinjected DNA from the cytoplasm [Lechardeur *et al.*, 1999].

Digestion of plasmid DNA by purified cytosol, obtained by selective permeabilization of the plasma membrane of HeLa cells, was divalent-cation dependent and thermosensitive, confirmed by Southern blotting and ^{32}P-release of end-labeled DNA [Lechardeur *et al.*, 1999]. The activation and inhibition profiles of the cytosolic nuclease are distinct from both that of apoptotic nucleases and DNase I or DNase II [Lechardeur *et al.*, 1999]. Thus the identity of the cytosolic nuclease(s), responsible for plasmid DNA degradation in the cytoplasm, remains to be established.

III) NUCLEAR TRANSLOCATION

The nuclear envelope is the ultimate obstacle to the nuclear entry of plasmid DNA. The inefficient nuclear uptake of plasmid DNA from the cytoplasm was recognized more

than twenty years ago. Comparison of the transfection efficiency of plasmid DNA encoding the thymidine kinase, introduced either into the cytosol or the nucleus, showed that not more than 0.1-0.001 % of the cytosolically injected plasmid DNA could be transcribed [Capecchi, 1980]. Similar results were obtained by injection of the ß-galactosidase reporter gene detection of radioactive or fluorescent plasmid DNA [Dowty *et al.*, 1995; Pollard *et al.*, 1998].

The Nuclear Envelope

Nucleocytoplasmic transport of macromolecules through the nuclear membrane is a fundamental process for the metabolism of eukaryotic cells. The trafficking of proteins and ribonucleoproteins is controlled by the nuclear pore complexes (NPCs) forming an aqueous channel through the nuclear envelope [Laskey, 1998]. While molecules smaller than ≈40 kDa can diffuse through the NPC passively, plasmids and other macromolecules larger than 60 kDa must comprise of a specific targeting signal, the nuclear localization sequence (NLS) to traverse the NPC in an energy-dependent manner [Talcott and Moore, 1999]. The diameter of the NPC channel reaches a maximum of ≈ 25 nm during active translocation, but the channel has a cross section of 9 nm when it is engaged in passive transport. The dynamic behavior of the NPC indicates that specific transport signals provoke considerable conformational change in the NPC. This provides a plausible explanation for the ability of the NPC to translocate substrates as large as 25-50 MDa [Harel and Forbes, 2001; Kuersten *et al.*, 2001]. Proteins or other cargo molecules that carry a NLS are recognized by the importin-α adapter, which in turn form complexes through the IBB (importin-β binding) domain with importin-β. Following the nuclear uptake of the complex through the NPC, association of Ran-GTP triggers the release of the imported polypeptides from importin "Fig. (3)".

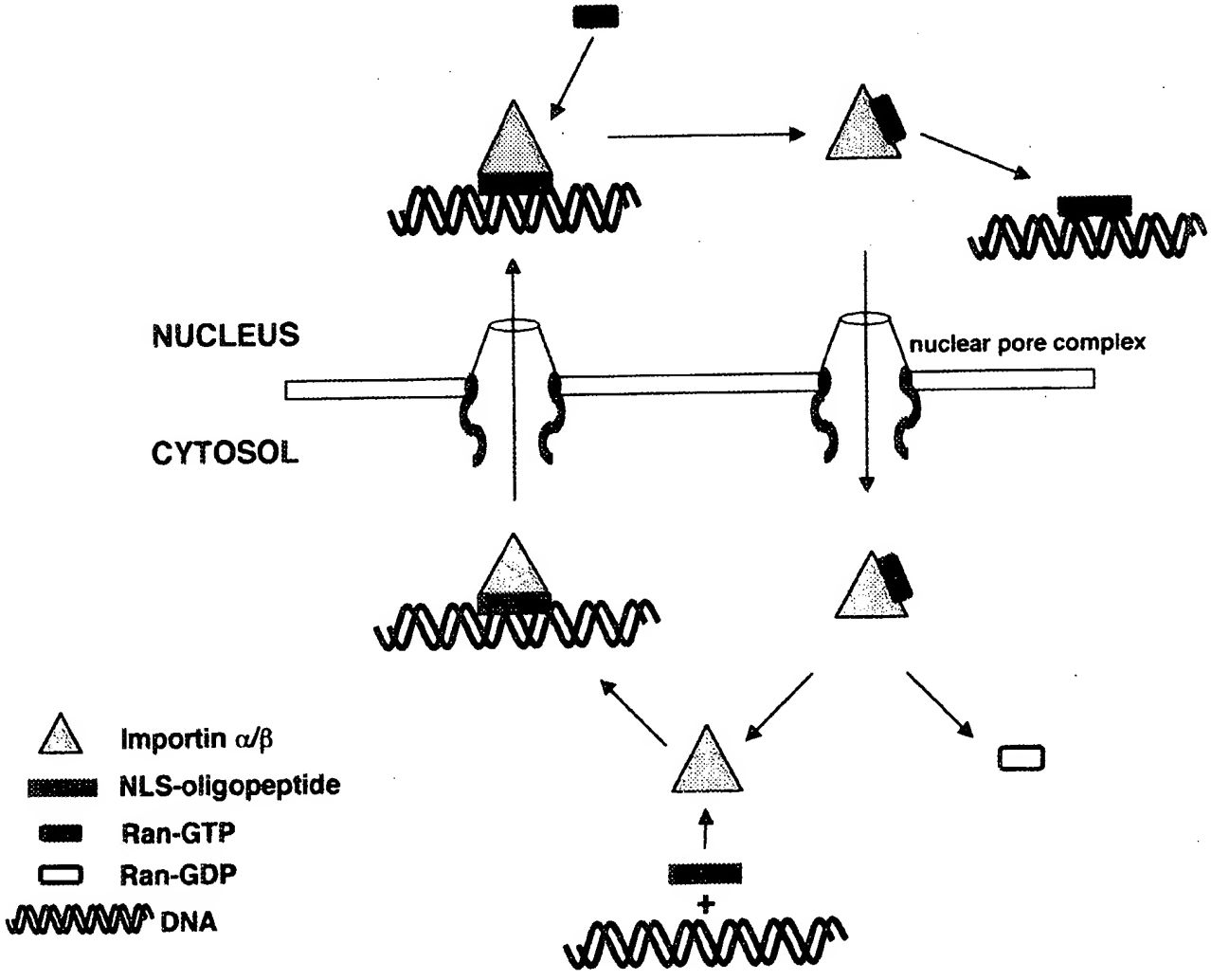

Fig. (3). Hypothetical mechanism of nuclear import of plasmid DNA by importin transport receptors. DNA is covalently attached to a NLS or bound to a NLS containing protein, such as transcription factor. The complex binds to importin in the cytoplasm and translocates into the nucleosol. Following nuclear entry, the importin-Ran GTP complex is recycled back to the cytoplasm where Ran-GTP is displaced from the complex upon the hydrolysis of GTP.

Nuclear Delivery of Plasmid DNA

The significant size of plasmid DNA (2-10 MDa) makes it unlikely that nuclear entry occurs by passive diffusion. The higher transfectability of dividing cells, compared to quiescent ones, suggested that plasmid DNA enter the nucleus preferentially upon the disassembly of the nuclear envelope during mitotic cell division [Brunner *et al.*, 2000; Mortimer *et al.*, 1999; Wilke *et al.*, 1996]. Meanwhile, accumulating evidence indicates that plasmid DNA can permeate the NPC by a mechanism that is reminiscent of the active transport of polypeptides larger than 60 kDa.

Blocking the cell cycle in the G1 phase by aphidicolin had no effect on the rate of internalization of lipoplex or on the level of transgene expression in stably transfected cells, but dramatically reduced reporter gene expression as compared to asynchronous cells [Mortimer *et al.*, 1999]. In addition, higher level of gene expression was observed when the cells were exposed to lipoplexes just before or during mitosis [Brunner *et al.*, 2000]. The slow proliferation rate is responsible, at least in part, for the limited efficiency of lipid-mediated gene transfer of primary cultures of ciliated human airway epithelia [Fasbender *et al.*, 1997b], in line with the notion that the disassembly of the nuclear envelope facilitates heterologous gene expression. In contrast, detection of gene expression of cytoplasmically microinjected reporter plasmid in primary myoblasts implies that plasmid DNA can enter postmitotic nuclei by a process sensitive to temperature and wheat germ agglutinin (WGA), a relatively specific inhibitor of the NPC-dependent active transport [Dowty *et al.*, 1995]. In the same work, gold labeled plasmid DNA was visualized by electron microscopy in the vicinity of NPC or inside the nucleus. The temperature-sensitive, energy-dependent and WGA-inhibitable nature of the nuclear translocation of plasmid DNA was confirmed recently, supporting the hypothesis that plasmid molecules can penetrate the nucleus through the NPC by an active mechanism [Brisson and Huang, 1999].

It is widely accepted that the size of expression cassettes constitutes a major impediment to nuclear targeting. The therapeutic potential of antisense oligonucleotides has prompted extensive studies of their intracellular trafficking. It has been shown that cytosolic microinjection promotes the rapid and preferential accumulation of oligonucleotides in the nucleus [Leonetti *et al.*, 1991]. Fluorescently tagged 15 to 25 bp oligomers could be detected in the nucleus within seconds after microinjection at 22 °C [Lukacs *et al.*, 2000]. Nuclear targeting of small size DNA (< 100 bp) was shown to be independent of the temperature, cytosolic ATP levels and the concentration of competing non-labeled oligonucleotide [Leonetti *et al.*, 1991]. These data, collectively, suggest that DNA fragments diffuse passively into the nucleus if their size is permissive (e.g. 20 bp double stranded oligomer is approximately equivalent in size to a 13 kD polypeptidede). The lack of binding of oligonucleotides to cytosolic polypeptides rules out the involvement of cytoplasmic factors in the nuclear import of small DNA fragments [Leonetti *et al.*, 1991]. The avid nuclear targeting and retention of oligonucleotides could be explained by the high number of non-specific nuclear binding sites [Clarenc *et al.*, 1993]. Comparison of diffusional mobility of DNA fragments in the cytoplasm and in the nucleus has revealed that oligonucleotides are poorly mobile in the nucleus [Lukacs *et al.*, 2000]. These observations indicate that due to their small size, oligonucleotides efficiently escape the transport barriers of the cytoplasm and nuclear envelope.

Attachment of NLS to plasmid DNA and DNA fragments stimulate both the nuclear accumulation and expression of plasmid DNA, consistent with the notion that DNA molecules can traverse the NPC [Branden *et al.*, 1999; Ludtke *et al.*, 1999; Sebestyen *et al.*, 1998; Wilson *et al.*, 1999; Zanta *et al.*, 1999]. Coupling of single or multiple classical NLS (SV-40 T antigen type) augmented the transfection efficiency, presumably, via the importin-dependent nuclear transport pathway. Similar if not more pronounced effect was observed by utilizing the non-classical NLS (M9 sequence of the human heterogenous nuclear ribonucleoprotein A1), enhancing the transfectability of non-dividing endothelial cells [Subramanian *et al.*, 1999]. While condensation of plasmid DNA by the positively charged linker peptide, comprising the NLS, may account, in part, for the effect, the majority could be attributed to the activity of the NLS. Replacing critical amino acid residues in the NLS abolished the effect of the targeting peptides [Zanta *et al.*, 1999].

The possibility that nuclear entry of plasmid DNA would be sequence dependent, depending on the binding of cytoplasmic factors encompassing a NLS, (e.g. transcription factors), has been examined [Dean, 1997; Dean *et al.*, 1999; Wilson *et al.*, 1999]. Engineering binding sites for endogenous transcription factor on the non-coding region of plasmid DNA have demonstrated that association of transcription factor may potentiate the expression of reporter molecules in cell specific manner [Vacik *et al.*, 1999].

Although direct comparison of the efficacy of synthetic peptides and transcription factors is not feasible, these results suggest that combination of transportin-, importin- and transcription factor-dependent nuclear targeting may have an additive effect on the nuclear uptake capacity of the non-viral delivery system. These experiments have not only verified that plasmid DNA can enter the nucleus by active translocation via the NPC in non-mitotic cells, but offer innovative solutions to overcome the cellular barrier to non-viral gene delivery as well.

IV) SOME OF THE STRATEGIES DEVELOPED BY VIRUSES TO OVERCOME CELLULAR BARRIERS

Viral particles can be large complexes up to a hundred nanometers in diameter [Kasamatsu and Nakanishi, 1998]. Therefore the movement of viruses in the cytoplasm and the nuclear delivery of their genome is unlikely to occur by passive diffusion. Despite the large size of their DNA, most of the viruses have the potential to target their genome efficiently into the nucleus. Recent studies using real-time video-image analysis of the infection pathway of single adeno-associated virus have demonstrated that the binding of a single virus particle to the cell membrane is sufficient to infect the host cell [Seisenberger *et al.*, 2001]. Incoming viruses can enter cells by endocytosis (adenovirus) or by

direct fusion of the viral membrane with the plasma membrane (Herpes Simplex Virus). Prior to the replication of the viral genome, most of the viral particles are subjected to a highly regulated uncoating, which culminates in the release of the viral genome in the cytosol. Viruses replicating in the nucleus have evolved to harbor escape mechanisms to overcome those cellular barriers that impede the nuclear delivery of plasmid DNA. In the following paragraphs, some of these viral strategies are discussed, which could serve as models to improve the nucleo-cytoplasmic transport of non-viral vectors "Fig. (4)".

Depending on the mechanism of infection, initial uncoating of the viral particle takes place in a pH dependent manner in endosomes and/or in the cytosol. Since the transport of viruses is highly efficient and rapid in the cytoplasm, it has been suspected that viruses rely on the cytoskeletal network during their vectorial movements inside the cell. This notion has been confirmed for several viruses, including Herpes Simplex Virus, Simian virus 40 and adenovirus. Herpes simplex virus enters the cell by direct fusion with the plasma membrane. Once in the cytosol, the virus moves along the MTs to the microtubule organizing center (MTOC) in ATP- and cytosol-dependent manner [Sodeik, 2000; Sodeik *et al.*, 1997]. Subsequent to the internalization of adenovirus, the capsid can escape the endosome and moves along the MTs toward the MTOC using the minus end-directed motor complex dynein/dynactin [Ploubidou and Way, 2001; Sodeik *et al.*, 1997; Suomalainen *et al.*, 1999]. The efficient intracellular transport of the capsid highlights the capacity of viral DNA to bypass the diffusional barrier of the cytoplasm.

The nuclear delivery of the genomic material of DNA viruses, which can exist in different conformations; -linear (adenovirus) or circular (papovavirus) double-stranded, partially circular double-stranded (hepadnaviruses) or linear single-stranded (parvovirus) -, is indispensable for productive infection "Fig. (4)". The size of the viral DNA is extremely heterogenous, ranging from 2.5 kb (e.g. Hepatitis B virus) to 150 kb (e.g. Herpes Simplex Virus), impeding the nuclear uptake of the viral genome. While some of the viruses have developed mechanisms to deliver their genome through the nuclear pore complex, others (e.g. retroviruses) require the breakdown of the nuclear membrane during mitosis [Kann *et al.*, 1997; Kasamatsu and Nakanishi, 1998]. The small DNA virus SV 40 is taken up by the nucleus via the NPC, following the unmasking of critical NLSs in the viral

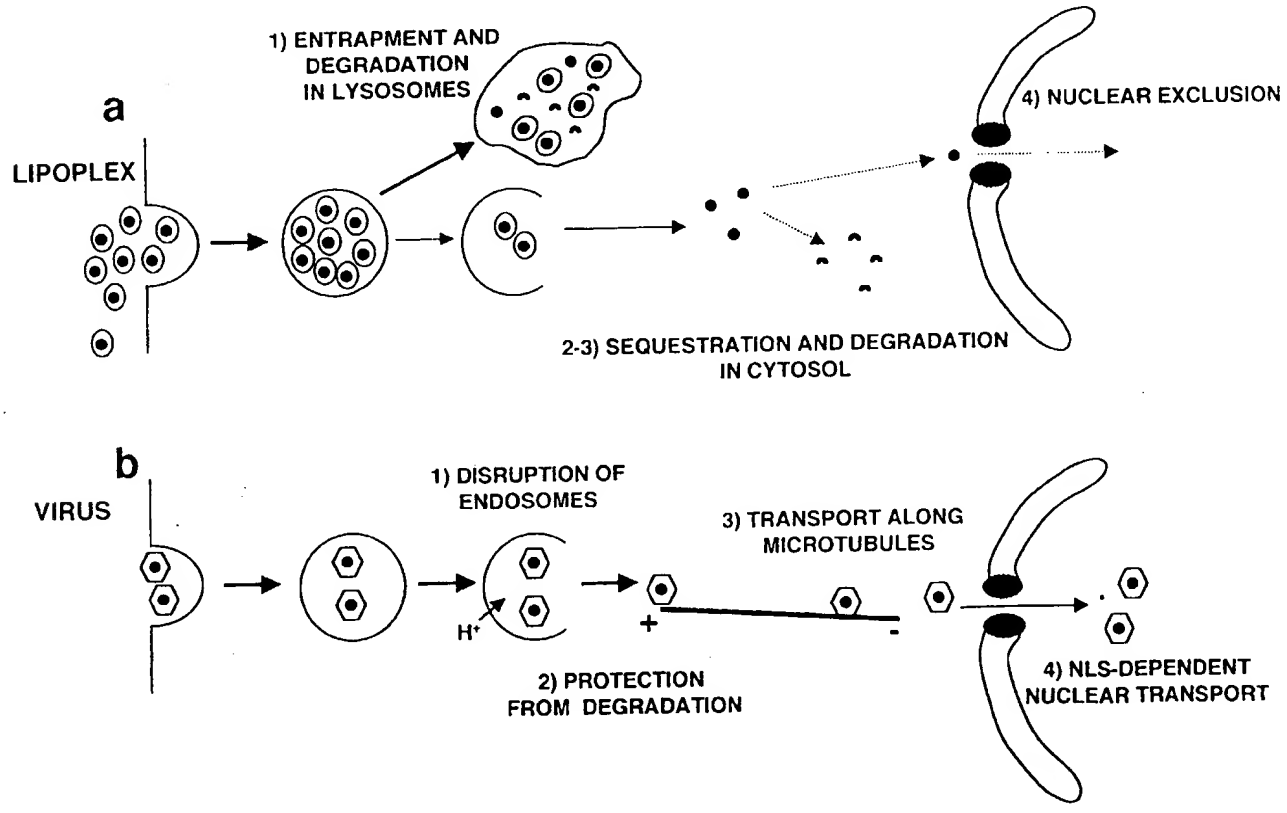

Fig. (4). Comparison of cellular trafficking of plasmid and viral DNA. A) Plasmid DNA complexed by synthetic vectors is successively trapped and degraded in the endo-lysosomes then in the cytoplasm before reaching the nuclear membrane. The size and the metabolic instability of plasmid DNA hampers efficient nuclear delivery. B) A number of DNA viruses are endocytosed via clathrin-coated vesicles. The virus uncoats and triggers the disruption of the endosome, leading to the cytosolic release of the viral DNA associated with some of the capsid proteins, protecting the DNA from degradation and facilitating transport to the nuclear envelope. Viral proteins bearing NLS targeting, recruitment of host proteins with NLS, or direct attachment of the viral genome to the nuclear pore complex ensure the efficient nuclear uptake of viral DNA.

proteins upon the conformational change induced by the acidic environment of endosomes. In this case, the ultimate uncoating of the DNA occurs in the nucleus [Greber, 1997; Greber and Kasamatsu, 1996; Nakanishi *et al.*, 1996]. In contrast, larger DNA viruses, such as the adenovirus or herpesvirus are submitted to a more intensive uncoating within the endosomes and/or the cytosol.

The nuclear import mechanism of adenovirus DNA has been recently elucidated. The adenovirus capsid, attached to the viral DNA binds to the CAN/Nup214, one of the filament proteins of the NPC. The nuclear import step of the DNA is mediated by the binding of the nuclear histone H1 to the capsid and proceeds in conjunction with H1-import factors [Harel and Forbes, 2001; Suomalainen *et al.*, 1999; trotman *et al.*, 2001]. These results with the observations that viral DNA, stripped from associated polypeptides, is unable to enter the nucleus, following microinjection or addition to digitonin permeabilized cells, underline the pivotal role of associated polypeptides in the highly efficient targeting process of the viral genome [Kann *et al.*, 1997; Mirzayans *et al.*, 1992].

V) CONCLUSIONS AND PERSPECTIVES

During the past decade it became evident that extracellular as well as cellular barriers, with tissue and organ specific characteristics, compromise the transfection efficiency of non-viral vectors. While the original interpretation emphasized the role of nuclear envelope as one of the major cellular barriers, recent data suggest that restricted mobility as well as metabolic instability of plasmid DNA, in concert with the nuclear barrier, contribute to the limited transfection efficiency of plasmid DNA. The challenge for gene therapy research is to pinpoint the rate limiting step(s) in this complex process and implement strategies to overcome the biological, physico-chemical and metabolic barriers encountered by therapeutic plasmid DNA during nuclear targeting.

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ABBREVIATIONS

DOPE = Dioleoylphosphatidylethanolamine

MT = Microtubule

MTOC = Microtubule-organizing center

NPC = Nuclear pore complex

NLS = Nuclear localization sequence

PEI = Polyethylenimine

WGA = Wheat germ agglutinin

NPC = Nuclear pore complex

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Synthetic peptide-based DNA complexes for nonviral gene delivery

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Abstract

A major advantage of synthetic peptide-based DNA delivery systems is its flexibility. By design, the composition of the final complex can be easily modified in response to experimental results in vitro and in vivo to take advantage of specific peptide sequences to overcome extra- and intracellular barriers to gene delivery. The extreme heterogeneity which greatly complicates both the kinetics of DNA–poly(L-lysine) interaction and the thermodynamic stability of the final DNA complexes is avoided. Other unique features include the absence of biohazards related to the viral genome as well as the production of the viral vector and the absence of limitations on the size of the therapeutic genes that can be inserted in the recombinant viral vector. In principle, if the gene can be cloned into an expression plasmid, it can be delivered as a synthetic DNA complex. Since these synthetic delivery systems are composed of small peptides which may be poorly antigenic, they hold the promise of repeated gene administration, a highly desirable feature which will be important for gene targeting in vivo to endothelial cells, monocytes, hepatocytes and tumor cells. © 1998 Elsevier Science B.V.

Keywords: Poly(L-lysine); Somatic gene therapy; Oligopeptides; Gene expression; Plasmid condensation; Receptor-mediated delivery

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1. Introduction

In 1995, an expert panel [1] commissioned by the National Institutes of Health evaluated the current state of gene therapy and described it as "a logical and natural progression in the application of fundamental biomedical science to medicine [with] extraordinary potential, in the long-term, for the management and correction of human disease, including inherited and acquired disorders." They also reported that "Significant problems remain in all basic aspects of gene therapy, [including] shortcomings in all current gene transfer vectors and an inadequate understanding of the biological interaction of these vectors with the host." The panel recommended "greater focus on basic aspects of gene transfer and gene expression [by] improving vectors for gene delivery, enhancing and maintaining high level expression of genes transferred to somatic cells, achieving tissue-specific and regulated expression of transferred genes, and directing gene transfer to specific cell types." The present review is focused on improved nonviral vectors for gene delivery that are peptide based.

1.1. Viral gene delivery

The principal strategies for gene delivery involve viral-mediated, chemical and physical mechanisms [2]. Viral vectors are widely used since the molecular biology of recombinant viruses is well understood and they give high level of DNA uptake and expression. However, major disadvantages of viral vectors for gene transfer include a lack of cell type specificity, a restriction in the size of DNA that can be used, safety considerations, and host immune response. For example, adenovirus-mediated gene delivery is highly efficient and can be used to transduce nondividing cells. There are, however, highly efficient host immune responses, which have evolved to contain viral infections, that limit the usefulness of the recombinant adenovirus. The cells that contain the exogenous gene of interest are destroyed by a cellular immune response directed against the viral gene products that are also expressed in the target cells. Viral proteins stimulate neutralizing antibodies, so that the gene delivery system can only be used once in a given individual.

1.2. Chemical nonviral DNA delivery

The complex and redundant anti-viral defenses have stimulated interest in alternative approaches such as nonviral DNA delivery vehicles. The objective of these research efforts is to construct self-assembling DNA complexes using synthetic chemical components that perform the roles of proteins in the viral vectors. The most promising delivery systems include complexes of DNA with positively charged polymers, lipids and synthetic peptides. The development of these synthetic delivery systems are at a very early stage, compared to the recombinant viruses. This review is limited to DNA plasmid delivery using synthetic peptides.

The ideal nonviral DNA delivery system should have the following properties (Fig. 1): (a) structurally well characterized, nontoxic, biodegradable, and nonantigenic systems that protect DNA from degradation and are stable in biofluids; (b) cellular uptake mediated by cell specific plasma membrane recep-

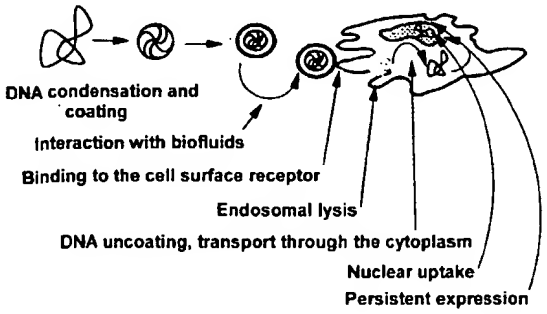


Fig. 1. Sequence for targeted delivery of a plasmid to a cell.

tors; (c) rapid pH dependent release from the endosome; (d) efficient dissociation of the DNA from the complex into the cytoplasm for transport of the DNA to the nucleus; (e) controlled duration and magnitude of expression.

2. Poly(L-lysine)-based gene delivery systems

2.1. Receptor-mediated gene delivery

The observation that cells have unique surface receptors provided an initial strategy to achieve specific delivery of genes to the liver (see Wu, chapter XX). The asialoglycoprotein receptor is a part of the surveillance system in the circulation that removes proteins as they age by the spontaneous loss of sialic acid [3,4]. This highly efficient hepatic receptor has been used by Wu and associates [5,6] to deliver reporter genes to the rat liver in vivo. Their experimental strategy involves covalently linking asialoorosomucoid (ASOR), a ligand for the asialoglycoprotein receptor, to poly(L-lysine). Poly(L-lysine) provides a positively charged template to which the negatively charged DNA expression vector binds through electrostatic interactions [7]. After hepatic uptake of the complex of DNA and the ASOR–poly(L-lysine) conjugate, the reporter gene product is found in the liver [6]. At present, experiments using direct in vivo delivery of DNA complexes to the liver [8–13] have produced only low numbers of transduced cells and small amounts of gene products. Other examples of receptor-mediated gene delivery include the transferrin receptor [14], epidermal growth factor EGF receptor [15], polymeric immunoglobulin receptor [16], CD3-T cell receptor [17], lectins [18,19], folate receptor [20], malarial circumsporozoite protein receptor [21], integrins [22], α_2-macroglobulin receptor [23], mannose receptor [24], the c-kit receptor [25], the insulin receptor [26,27], the thrombomodulin receptor [28], surfactant protein A and B [29,30] and the mucin receptor [31].

2.2. Advantages and disadvantages

The attractive feature of receptor-mediated gene delivery is that it provides an opportunity to achieve cell specific delivery of DNA complexes. The structure–function relationships for many receptor ligands are known, so it should be possible to obtain receptor ligands with high binding affinities (1–10 nM). Most of the receptor ligands are proteins and in many cases, the receptor binding ligand domain of the protein has been identified by site specific mutagenesis. In some cases, the distribution of specific receptors in various organs and tissues has been determined. The mechanisms and routes of internalization of several receptor–ligand complexes in vivo have been partially characterized, so that the biodistribution of the DNA complex can be predicted, if it has the biochemical characteristics of the native ligand.

There are several formidable obstacles to the routine use of receptor-mediated gene delivery. The naturally occurring receptor ligands are either proteins or complex carbohydrates, which are extremely difficult to obtain consistently in high purity ($>98\%$) and in sufficient quantity. These receptor ligands are usually covalently crosslinked to poly(L-lysine), thereby creating novel antigenic epitopes [11]. Since the crosslinking reagents are nonspecific, a random complex mixture of receptor–ligand–poly(L-lysine) conjugates are produced [32,33]. Because the conformation of the binding surface is formed by chance, binding of DNA to the conjugates is variable.

The chain length heterogeneity of the commercially available poly(L-lysine) is one of the major causes of variability in the formulation of reproducible, stable formulations. The poly(L-lysine) is synthesized by polymerization of the N-carboxy-anhydride of lysine, fractionated and characterized in terms of the

average degree of polymerization and average molecular weight. Dolník and Novotny [34] derivatized the amino groups of the 3.5, 17.2 and 40–60 kiloDaltons (kDa) poly(L-lysine) preparations with 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde and separated the mixtures by capillary polyacrylamide gel electrophoresis. As expected from the synthetic method for the polymer, the derivatized poly(L-lysine) samples were extremely heterogeneous. The 3.5 kDa preparation, described as 17 for the degree of polymerization, had 13 separate peaks, only one of which contained 17 lysine residues. From inspection of the chromatographic profile illustrated in the paper, about 75% of the mass in the heterogeneous mixture was found in six peptides, which were present in roughly equal proportions. More than 45 oligopeptides are present in the 71.2 kDa sample, designated as 82 for the degree of polymerization. Thus, the polydispersity of commercial poly(L-lysine) means that the individual molecular species of the polycation interact with DNA with individually distinct kinetics, for both the electrostatic and the hydrophobic interactions. The extreme heterogeneity greatly complicates both the kinetics of DNA–poly(L-lysine) interaction and the thermodynamic stability of the final DNA complexes. Further, poly(L-lysine) exists as a random-coil at neutral pH, an alpha-helix at alkaline pH and a mixture of conformations at pH 7.4 [35].

The difficulty of using polydisperse poly(L-lysine) in formulations of DNA complexes has been documented with atomic force microscopic analysis [36]. Expression vectors 6 kilobase (kb) were condensed with poly(L-lysine) with different degrees of polymerization, with molecular weights that ranged from 3.9 to 224.5 kDa. The highest molecular weight poly(L-lysine) preparation produced large complexes with significant polydispersity (diameters ranging from 120–300 nm), while the smallest poly(L-lysine) produced more homogeneous complexes with diameters ranging from 20–30 nm. Other poly(L-lysine) preparations of molecular weight 53.7 and 23.8 kDa produced complexes of intermediate size and polydispersity. The mean volumes of the complexes formed using poly(L-lysine) 2245 and 3.97 kDa were 606 000 nm^3 and 3700 nm^3, respectively.

In addition to the molecular heterogeneity of these poly(L-lysines), they are toxic to living cells in nM concentrations, which limits their general applicability. Gottschalk et al. [37] synthesized a short poly(L-lysine) analog, $YKAK_8WK$ (K8), which contains a central cluster of eight lysines. To show that K8 is not cytotoxic to cells, HepG2 cells were incubated at 37°C for 24 h with increasing concentrations of K8 or poly(L-lysine) (100 mer), after which viable cells were counted. Poly(L-lysine) concentrations of greater than 0.1 μM led to complete cell death. By contrast, no cytotoxicity was observed for up to 100 μM of K8, the highest concentration tested. This comparison indicates that K8 is at least 1000-fold less toxic than poly(L-lysine) for HepG2 cells.

The variable stoichiometry of the components in the complex has made it difficult to prepare the complexes either consistently well or in sufficient quantity for in vivo delivery [33,34] and precludes a molecular definition of the biologically active reagent.

3. Dynamics of DNA condensation

Since DNA condensation is a critical process for the synthetic DNA delivery systems, it is useful to summarize several studies that describe the dynamics of DNA condensation. These studies of the interactions of DNA with small molecules have yielded mechanistic insight in the kinetics and thermodynamics of DNA condensation by cations. The descriptions of these mechanisms and of the kinetic dominance of the assembly processes for the DNA complexes provide a useful perspective in which to consider the interaction of DNA with cationic oligopeptides, until which time comparable studies are available for the reagents used for gene delivery. It is likely that the mechanisms for DNA interaction with metal ions, spermine and cationic oligopeptides will have much in common.

The focus on the size of the DNA delivery system and the physicochemical processes that control its stability comes from the recognition that the dimensions of the clathrin-coated pit places an upper limit on the size of DNA complexes that can undergo endocytosis [38]. Freeze–fracture electron micrographs show diameters of clathrin coated pits as 100–200 nm [39]. The kinetics of dissociation of the DNA–polycation complex after cellular uptake are

also critically important. It is not known if the rate of endosomal and/or cytoplasmic enzymatic degradation [40] of poly(L-lysine) in the complex is important for transit to the nucleus. If the affinity of the DNA binding template is too low, the DNA complex will dissociate prematurely. If the affinity is too high and/or the rate of enzymatic cleavage in the cytoplasm is too slow, the size and shape of the DNA torus will prevent movement of the DNA through the cytoplasm and transport through the nuclear pore [41–45].

3.1. Interaction of DNA with spermine and other small cations

Wilson and Bloomfield [46] studied condensation of DNA from its extended coil form to a condensed form by spermidine, spermine and other cations. Using Manning's [47] counterion condensation theory, they calculated a unity among these disparate ions: the collapse occurs in each case when from 89 to 90% of the DNA phosphate charges are neutralized by condensed counterions.

Bloomfield [48] notes that the DNA condensate size distribution is independent of the length of the DNA molecule, from 400 to 40 000 basepairs (bp). To explain these results, an equation devised by Tanford for micelle formation was adapted. In this model, most of the obvious attractive and repulsive free energy contributions (mixing, bending, hydration, and other nearest-neighbor interactions) are linear in the amount of DNA incorporated, but the net attractive ΔG°, the standard free-energy change, grows nonlinearly because of the increasing average number of nearest neighbors of each duplex as the particle grows. It appears that the size distribution of condensed particles is determined kinetically rather than thermodynamically. A constant radius of curvature model for the organization of DNA in toroidal condensates has been developed [49]. The kinetic model for the toroid formation process predicts a distribution of toroid sizes for DNA condensed from solution that is in good agreement with experimental data. Slow condensation kinetics may be required to overcome the high activation energy of highly distorted DNA bends or kinks at the turning points of rods. Condensation may also be associated with localized helix structure distortion provoked by condensing agents.

Porschke [50] investigated the condensation of DNA induced by spermine and spermidine using equilibrium titrations, stopped–flow and field–jump experiments with scattered light detection. Stopped–flow measurements of the spermine-induced condensation demonstrated the existence of two processes: a "fast" reaction was observed in the millisecond time range, when the reactant concentrations were around 1 μM. The fast process was associated with a characteristic induction period and assigned to the intramolecular condensation reaction. A slow reaction with time constants of about 100 s was strongly dependent upon both spermine and DNA concentrations. The second process was assigned to an intermolecular DNA association. The unusual time course of the intramolecular condensation reaction with the induction period provides evidence for "threshold kinetics". During the induction period, spermine molecules are bound to DNA, but the degree of binding remains below the threshold value. As soon as the degree of ligand binding arrives at the threshold, the DNA is condensed in a relatively fast reaction. Model calculations of the spermine binding kinetics according to an excluded-site model demonstrate that the spermine molecules bound to DNA are mobile along the double helix. A comparison of the experimental data with the results of Monte Carlo simulations suggests a rate constant of approximately 200 s^{-1} for spermine movement by one nucleotide. The slow process appears to be associated with localized distortions of helical structures.

Honig and coworkers [51] note that, in addition to salt-dependent electrostatic effects, the ion atmosphere and dielectric effects are a major factor in determining the stability, structure, reactivity, and binding behavior of nucleic acids. They envision the electrostatic binding free energy as a balance between the coulombic attraction of a ligand to DNA and the disruption of solvent upon binding [52]. The formation of a ligand–DNA complex removes both charged and polar groups at the binding interface from pure solvent, while binding displaces salt from around the nucleic acid. As a result, the total electrostatic binding free energy is quite small. Consequently, nonpolar interactions, such as tight

packing and hydrophobic forces, must play a significant role in ligand–DNA stability. Westhof [53] reviews water as an integral part of nucleic acid structure. Billeter [54] notes that hydration water molecules are known to play an important role for the folding, the stability and the function of biological macromolecules. Thus, the width of the minor groove in B-DNA correlates with the order and the lifetime of water molecules observed in this groove.

Recent progress includes the observation of the collapse of single DNA molecules, greater insights into the intermolecular forces driving condensation, the recognition of helix-structure perturbation in condensed DNA, and the increasing recognition of the likely biological consequences of condensation [55,56].

3.2. Interaction of oppositely charged polymers

The interaction of DNA with small polycations is one of the most critical steps in the formulation procedures for peptide based DNA delivery systems. The review by Kabanov and Kabanov [57] provides an excellent and relevant perspective on the interaction of oppositely charged polymers. Upon mixing, DNA, a negatively charged polymer, spontaneously assembles with poly(L-lysine), a linear polycation, as the result of the formation of a cooperative system of interchain electrostatic bonds. These authors note that the physicochemical characteristics of the DNA complexes, specifically their solubility, dimensions, and surface charge, are determined by the chemical composition of the reactants. Whether the DNA precipitates when mixed with the polycation or forms a soluble complex depends on pH, ionic strength, temperature, medium composition, concentration of reactants, and, most importantly, the molecular homogeneity of the reactants.

The kinetics of DNA–poly(L-lysine) association, while diffusion controlled, are complex for several reasons. The molecular weights of DNA and poly(L-lysine) are different by two to three orders of magnitude. The molecular weight of the recombinant DNA molecule can range from about one million to tens of million Daltons. By contrast, the molecular mass of poly(L-lysine) ranges from ~1 kDa to several hundred thousand kDa. Thus, in the guest–host terminology for interacting oppositely charged polymers [57], DNA can be termed the host for the smaller molecular weight poly(L-lysine) guest. The contour length of the poly(L-lysine) is at least 100 times less than that of the DNA. The poly(L-lysine) chains are probably evenly distributed along the DNA molecules because of the mobility of the poly(L-lysine) [57]. This movement along the surface of the DNA would require significantly less energy expenditure than would be required for dissociation and rebinding, since both of the latter processes are dominated by changes in water structure.

Binding of polycations induces DNA compaction due to compensation of electrostatic charges of DNA and hydrophobic interactions of the complexed sites. The charge ratio in local regions of the polynucleotide changes over time as it becomes compact as the DNA complex forms. The polycation chains are not rigidly fixed on the DNA chains but migrate from one DNA helix to another, until the overall charge ratio of the complex is 1 or greater. Kabanov and Kabanov [57] note that when the backbone of the polycation is hydrophobic, as is the case with the amide backbone of poly(L-lysine), the association of the polycation with the nucleic acid and neutralization of the phosphate group charges, leads to the formation of hydrophobic sites. The length and number of hydrophobic sites depends on the length of the polypeptide and the charge ratio of the complex. These authors suggest that the amount of poly(L-lysine) that can be incorporated in a complex is limited by the number of binding sites on DNA which provide cooperative electrostatic binding of the polycation. The maximum length of such sites for cooperative binding of polyions is about 10 polyion units.

The process of DNA complex formation described by Kabanov and Kabanov [57] is the following. The interaction can then be envisioned as the following phases: (a) coating of the supercoiled DNA with the polycation; (b) migration of the polycation along the anionic helix; (c) formation of hydrophobic sites; and (d) positively charged poly(L-lysine) loops bound to DNA. The initial complexes are negatively charged and nonstoichiometric, i.e., more phosphates than ε-amino groups. With further incorporation of the polycation, there is an increase in the number of hydrophobic sites. The complex reaches a critical

composition, with an increased hydrophobicity such that disproportionation into two populations of non-stoichiometric (lysine < phosphate) and stoichiometric complexes (lysine = phosphate) occurs spontaneously. Depending on the number and location of hydrophobic sites, aggregation of the DNA–poly(L-lysine) complexes may be the dominant process. Upon the addition of more polycations, they are incorporated into the insoluble stoichiometric complexes. Further increases in the amount of polycation leads to dissolution of the stoichiometric complexes, which become positively charged particles, stabilized in solution by polycation loops bound to DNA.

Taking all of these considerations together, it is clear that mixtures of DNA complexes formulated with poly(L-lysine) are extremely complex. As noted previously, the polydispersity of commercial poly(L-lysine) endows individual molecular species of the polycationic mixture with individually distinct kinetics, for both the electrostatic and the hydrophobic interactions with DNA. The high molecular weight and variable stoichiometry of the complex of DNA and the receptor–ligand–poly(L-lysine) conjugates make it very difficult to prepare the DNA–receptor–ligand–poly(L-lysine) complexes either consistently well, or in sufficient quantities for in vivo delivery, and represent the inherent limitations of this method. The complexity of the mixtures precludes a molecular definition of the biologically active reagent. The heterogeneity of the mixtures most likely contributes significantly to the meager in vivo results.

4. DNA delivery using synthetic peptides

4.1. Rationale for synthetic peptide-based DNA delivery systems

The rationale for simpler synthetic systems for DNA delivery evolved from the biochemical knowledge that the active sites of enzymes, receptor ligands and antibodies involve about 5 to 20 amino acids. Thus, it should be possible to use small synthetic peptides to emulate the active sites of viral proteins and formulate synthetic DNA complexes that are as efficient as viruses, but do not have their limitations [2,58]. The advantages of synthetic peptides include the following: the molecular structure and purity of the reagents is readily and accurately determined. The synthetic approach is extremely versatile, with the capability to synthesize multifunctional reagents. This approach is necessary to provide a rational basis, rather than an empirical one, on which significant improvements can be made in the delivery systems. It will allow stabilization of the DNA complexes in various biofluids through systematic experimental changes in the composition of the complex and the conditions for formulation.

A major advantage of this approach is its flexibility. By design, the composition of the final complex can be easily modified in response to experimental results in vitro and in vivo. This design flexibility is important since there is little quantitative information about how efficiently the vector is processed through each stage of the complex processes of cellular uptake and transport to the nucleus. The processes that might account for the observed low efficiency of the existing methods of nonviral gene delivery remain to be identified. In addition to sequences for endosome lysis and nuclear uptake, there may be other essential but as yet unidentified functions of viral proteins. The stepwise assembly of DNA complexes using small peptides which are functional equivalents of the much larger more complex viral and cellular proteins should also improve our understanding of how viruses invade and replicate.

The principal advantage of using a synthetic peptide-based gene delivery system is the potential ability to take advantage of specific peptide sequences to overcome extra- and intracellular barriers to gene delivery. Specific sequences of interest for gene delivery include DNA binding and protecting peptides which dissociate in the cytoplasm, peptide ligands for receptor-mediated uptake, peptides with endosomolytic properties to release DNA from the endosomes, and peptides that facilitate nuclear transport of DNA.

While the ultimate objective is to design and assemble systems that have many of the properties of an ideal nonviral DNA delivery system (Section 1.2), the initial objective for our studies of peptide design focused on condensation of plasmid DNA. Further, in view of the existing literature on the use of poly(L-lysine) conjugates for DNA delivery, we reasoned that these heterogeneous systems could be

replaced with rigorously defined, molecularly homogeneous lysine-rich synthetic peptides.

4.2. Interaction of DNA with synthetic lysine containing oligopeptides

4.2.1. KYK and KWK

Studies of the kinetics of interaction between duplex DNA and lysine oligopeptides are limited to KYK and KWK, which Porschke and Ronnenberg [59] investigated by the field–jump method using fluorescence detection. Two separate relaxation processes, clearly distinguished on the time scale and by opposite amplitudes, are observed for the binding of KWK to both 30 kb DNA and ~500 bp sonicated DNA. The data were best described by a two-step association reaction, a mechanism with a bimolecular step followed by a slow intramolecular transition. The intramolecular event was assigned to an insertion of the aromatic residues into the DNA associated with bending of the helix. For binding of KYK to the same DNA, two processes could be identified and were assigned to a two step mechanism corresponding to that observed for KWK. The dissociation of the complex was rapid. The dissociation rate constant for the first step was $4 \times 10^3\ s^{-1}$, whereas it was $4.4 \times 10^2\ s^{-1}$ for the second step, which correspond to halftimes of 0.17 and 1.6 ms, respectively. As the reverse of the association steps, the slow step in dissociation of the DNA–WKW complex would first involve extraction of the aromatic nucleus from the DNA helix, followed by a rapid release of the cationic peptide from the DNA template. For reference, Porschke [50] found that the dissociation of polyamine–DNA complexes, which do not involve intercalated ligands, had time constants of 5 and 600 ms for release of spermidine and spermine, respectively. A priori, one would expect that the rate constants for disruption of electrostatic bonds between DNA and short lysine oligopeptides to decrease with increasing chain length. The difference may be more than tenfold, until the overall net charge difference between successive homologous peptides is small, i.e., less than 5%.

4.2.2. K_nWK-amides

Lohman et al. [60] studied pentalysine–DNA interactions as a model for the general effects of ion concentrations on the interactions of proteins with nucleic acids. The observed binding constant decreases dramatically with both increasing NaCl concentration and pH. The data are consistent with an electrostatic interaction between the cationic peptide and DNA, driven by the entropic contribution of counterion release to the free energy of binding. This work was extended with oligolysines containing tryptophan by examining the thermodynamics of binding of KWK_2–NH_2 and KWK_4–NH_2 with DNA [61]. As observed with pentalysine, the free energy of binding of the peptide amides was entirely entropic in origin. Identical binding behavior was observed for linear and negatively supercoiled plasmid. Quenching of tryptophan fluorescence was independent of the charge on the peptides. Recently, Zhang et al. [62] have used KWK_6–NH_2 to show that the polyelectrolyte character of a polymeric nucleic acid makes a large contribution to both the magnitude and the salt concentration dependence of its binding interactions with simple oligocationic ligands.

4.2.3. $YKAK_nWK$

The dissociation of KWK from a peptide–DNA complex, with a halftime of about 1.6 ms, appeared much too rapid to give a complex sufficiently stable for gene delivery. Since there is linear relationship of the binding constants for K_nWK-amides with nucleic acids [61], we reasoned that increasing the number of lysine residues in KWK would enable us to prepare DNA complexes which dissociated more slowly. We synthesized a series of peptides having the general structure of $YKAK_nWK$, where $n = 4$, 5, 6, 7, 8, 10 and 12 lysine moieties in the central cationic cluster. Orthogonal views of one of these peptides, $YKAK_8WK$ (K8), are shown in Fig. 2.

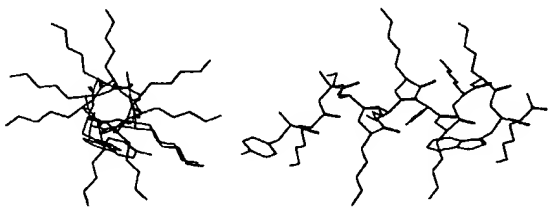


Fig. 2. $YKAK_8WK$ viewed from the end of the α-helix (left) and from the side (right).

To facilitate the characterization of the DNA complexes, as well as the quantification of the biodistribution of the complex in vivo, a tyrosine was added at the amino terminal of each peptide for ^{125}I- and ^{131}I-labels. The lysine at position 2 of the peptides was included as a branching residue for attachment of receptor ligands and reporter groups on its ε-amino group. Before the synthesis, molecular modeling indicated that the series of peptides would have an α-helical configuration. Since insertion of the tryptophan into the DNA helix [63] would provide orientation of the peptide at the surface of the DNA, an alanine residue was included in the sequence to place Lys2 and the tryptophan side chain on opposite sides of the putative helix.

The active gene delivery complex was constructed step-wise by spontaneous self-assembly processes involving oppositely charged, electrostatic interactions. For assembly of DNA–peptide complexes with different overall net charges, only the negative charges of DNA phosphate and the positive charges of the ε-amino groups of lysine in the $YKAK_nWK$ peptides were considered.

For the initial evaluation, DNA was combined with each of the $YKAK_nWK$ peptides in water. By dynamic light scattering, small DNA complexes (< 100 nm) were formed with all of the peptides at ratios of phosphate to lysine that ranged from 1:0.1 to 1:10, except at charge ratios of 1:1 where large aggregates (> 1000 nm) formed. These complexes were stable at room temperature for at least 5 days (Duguid, 1996; unpublished data). Combination of DNA and the $YKAK_nWK$ peptides in 0.15 M NaCl produced aggregates.

Of all the $YKAK_nWK$ complexes, only those containing eight or more lysines were active. $YKAK_8WK$ gave gene transfer that was about tenfold greater than that given by DNA only in HepG2 cells [37]. As is the case for DNA–poly(L-lysine) complexes, a lytic peptide is needed for efficient gene delivery (Section 4.3.3).

4.2.4. Alkylated CWK_n

Wadhwa et al. [64] describe a series of DNA condensing peptides which contained an amino terminal cysteine, followed by a tryptophan and then a lysine repeat containing 3, 8, 13 or 18 residues. Single chain reagents were produced by alkylating the cysteine with iodoacetic acid. Dimeric peptides were produced by oxidation of the cysteine. The most effective peptides in condensing DNA to 50–60 nm particles were the dimeric peptides containing 14 or more lysine residues. These positively charged particles were effective for transfecting HepG2 and COS 7 cells, with luciferase expression about 10^4 times greater than that produced by DNA only. Chloroquine was used as the endosomolytic agent in these experiments. Noteworthy is the direct comparison of alkylated CWK_{18} with the commercially available poly(L-lysine)$_{19}$. The homogeneous, single molecular entity, alkylated CWK_{18}, gave a particle with a mean diameter of 78±30 nm, while the polydisperse poly(L-lysine)$_{19}$ produced a condensate with a mean diameter of 3102±297 nm. By capillary electrophoresis, the poly(L-lysine)$_{19}$ contains more than 20 molecular species, with about six homologues being present in equal proportions [34]. Their electron microscopy studies [64] clearly confirm the light scattering data that the heterogeneous mixture of peptides in poly(L-lysine) produce even more complex aggregates of condensed DNA. The differences in gene delivery by alkylated CWK_{18} and poly(L-lysine)$_{19}$ were also impressive, with luciferase expression about 1000 greater in HepG2 with alkylated CWK_{18} as compared to the polymeric mixture.

4.3. Requirement for endosomolytic peptides

4.3.1. Recombinant adenovirus–poly(L-lysine) complexes

Because the poly(L-lysine)s are commercially available, complexes of DNA with ligand–poly(L-lysine) conjugates for cellular uptake via receptor-mediated endocytosis have been widely used. Toroid structures can be obtained which are small enough (80–100 nm) to be engulfed by endosomes [65]. However, these particles lack the ability to actively escape from the endosome before reaching the lysosomal compartment, which strongly decreases transfection efficiency. Therefore adenovirus particles have been incorporated to supply endosomal escape activity [66,67] to improve gene expression in vitro. One effect of adenovirus is to lyse the endosome before the contents can be either routed to the lysosomes or recycled to the cell surface. When

DNA–ligand complexes and adenovirus are co-internalized in vitro, it is possible to obtain 100% transduction and a high level of gene expression [67]. The size of the clathrin-coated pit constrains uptake of conjugates of adenovirus and a DNA complex, probably a limit of one virus in a DNA complex, a small proportion of the complexes observed by electron microscopy. These DNA–ASOR–poly(L-lysine)–adenovirus complexes fail to give expression in vivo after systemic injection, most likely because they are too large to pass though the fenestrations of the liver endothelium, which provides an additional mechanical barrier for the hepatocytes [68]. Further, particles larger than 100–110 nm are phagocytosed by the Kupffer cells. Apparently, this clearance supersedes potential targeting by receptor ligands. In addition, the immune response to the adenovirus prevents readministration of these hybrid molecules.

4.3.2. Endosomolytic peptides–poly(L-lysine) complexes

4.3.2.1. Influenza virus hemagglutinin peptides

An alternative approach to achieve endosome rupture without a viral component is lysis produced by specific peptide sequences in viral coat proteins. Short synthetic peptides containing the first 23 amino acids of the HA_2 subunit of influenza hemagglutinin protein (HA) have been studied extensively [69,70]. Although the rates are slower, these peptides give both fusion and leakage of liposomal contents, similar to that produced by whole HA molecules. HA peptides were also active in erythrocyte lysis. When these peptides were incorporated into DNA complexes by ionic interaction with positively charged polylysine–DNA complexes, a strong correlation between pH-specific erythrocyte disruption activity and gene transfer was observed [69,70]. Midoux et al. [71] have also utilized a 22-residue HA peptide to enhance the transfection activity of lactosylated poly(L-lysine)–DNA complexes. Kamata et al. [72] added amphiphilic peptides, GLFEAIAEFIEGGWEGLIEG and GLFKAIAKFIKGGWKGLIKG, to the medium at a concentration of 1 μM for transfection for several cell lines with Rous Sarcoma virus promoter-β-galactosidase-[N-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniumchloride-dioleoylphosphatidylethanolamine and found that the peptides gave a three- to fivefold enhancement of the Lipofectin-mediated transfection.

4.3.2.2. GALA

Parente et al. [73] have developed a model that can predict the leakage kinetics of solutes entrapped in lipid vesicles induced by a pore-forming peptide. The synthetic, amphipathic peptide GALA undergoes a pH-dependent conformational change and induces leakage of contents from large unilamellar phosphatidylcholine vesicles when in a helical conformation. The leakage depends on the size of the entrapped molecules and occurs by an all or none mechanism; vesicles either leak or retain all of their contents. In this model, GALA becomes incorporated into the vesicle bilayer and aggregates to form a pore. With a critical number of peptides, they assemble into a supramolecular aggregate as a transbilayer channel composed of 8–12 monomers. The channel diameter ranges from 5–10 Å. Other lytic peptides used for gene delivery have not been studied in this detail. It seems likely that the model is general and useful for defining the kinetics and properties of this class of peptides.

4.3.2.3. Gramicidin S

The combination of the cyclic cationic amphipathic peptide gramicidin S and dioleoylphosphatidylethanolamine gives transient expression levels of β-galactosidase at levels up to 20-fold higher than cationic liposomes in adherent mammalian cells [74]. Since transfection in CV-1 cells is not affected by lysomotrophic agents, DNA entry into the cell appears to be via the plasma membrane, rather than by endocytosis.

4.3.3. Endosomolytic peptides–$YKAK_8WK$ complexes

4.3.3.1. JTS-1, GLFEALLELLESLWELLLEA

We have designed from first principles an amphipathic membrane associating peptide, JTS-1 [37], shown in Fig. 3. The hydrophobic face contains only strongly apolar amino acids, while the hydrophilic face is dominated by negatively charged glutamic acid residues at physiological pH values. Molecular

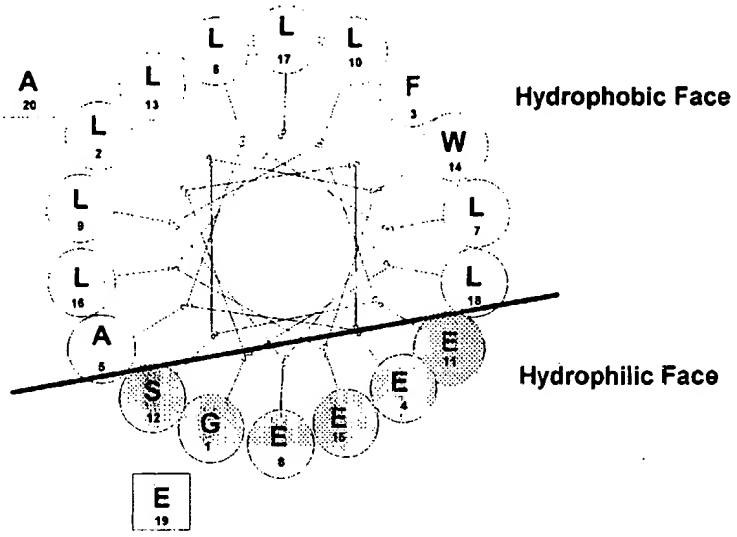


Fig. 3. Helical wheel of JTS-1.

modeling and structure prediction programs suggest that the hydrophobic face of the peptide causes self-association and forms pores in one side of the endosomal membrane, thereby destabilizing the membrane which leads to its rupture.

In forming an active gene transfer complex, only the negative charges of the five γ-carboxyl groups of JTS-1 were considered. The cationic DNA complex formed with K8 was rapidly mixed with negatively charged JTS-1, which was spontaneously incorporated through electrostatic interactions into the tertiary complex. Transfection using these complexes of cytomegalovirus promoter-luciferase, JTS-1 and K8 gave high levels of gene expression in 16 cell lines in vitro [37]. Using β-galactosidase expression as an index of transduction, the efficiency of delivery varied from 1–90% in about 20 different types of cultured cells. Typically, 10–40% of the cells are transduced. The human epithelial cell line 293 that has the adenoviral early genes EIA and E2 is transduced very efficiently with the peptide system.

4.3.3.2. *Analogs of YKAK$_8$WK*

The effects of alterations in the length of the lysine side chain of YKAK$_8$WK on DNA transfection in RAW264 cells has been studied systematically, by modifications of the central cationic cluster of the reference peptide [75]. The analogs were YKA(δ-O)$_8$WK (δ-O8) in which ornithine was substituted for lysine and the peptide bonds utilized the δ-amino group of ornithine; YKA(ε-K)$_8$WK (ε-K8) in which the peptide bonds utilized the ε-amino group of lysine; YKA(Dab)$_8$WK (Dab8) containing 1,4-diaminobutanoic acid; YKA(O)$_8$WK (O8) containing ornithine; YKA(R)$_8$WK (R8) containing arginine; D-YKA(K)$_8$WK (DK8) made with D-amino acids; and KWK$_5$C-CK$_5$WK [2(K$_5$C-)] a dimeric peptide containing a disulfide bridge. Each peptide was combined with JTS-1, in lieu of YKA(K)$_8$WK (K8) which served as reference for transfection efficiency using β-galactosidase in RAW264 macrophages. The results are shown in Fig. 4. The peptides were tested in RAW264 macrophages, using 20 μg β-galactosidase in complexes with 1:3:1 phosphate–lysine–glutamate ratio.

Transfection efficiency increases with the length of side chain spacer arm, i.e., transfection efficiency increases as δ-O8 = ε-K8 = Dab8 < O8 < K8). In the peptides, δ-O8 and ε-K8, the amides are formed with the δ-and ε-amino groups of ornithine and lysine. The dipeptide, 2(K$_5$C-), formed by linkage of the cysteine disulfide groups, enhances transfection significantly. This is most likely due to the increased ease of degradation of the peptide by reduction of the

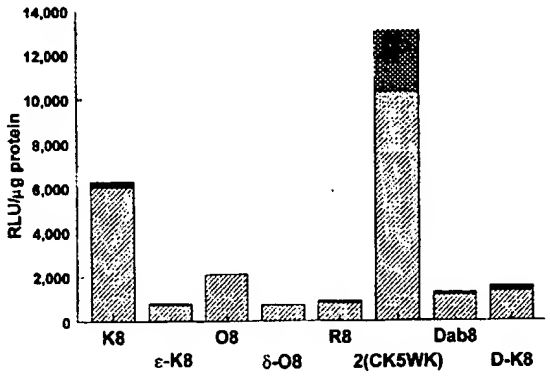


Fig. 4. Gene delivery with analogs of $YKAK_nWK$.

disulfide bonds and decreased DNA binding affinity of the resulting monomer peptide.

Gottschalk et al. [37] and Wadhwa et al. [64] both included tryptophan in their peptides for monitoring the interaction of the peptide with DNA by fluorescence quenching. To test the sequence specificity of $YKAK_8WK$, Duguid (1996; unpublished data) tested a series of peptides in which substitutions were made for tryptophan at position 12 (Fig. 5). The analogs of $YKAK_8WK$ include $phe^{12}K8$, $his^{12}K8$, $tyr^{12}K8$, $ala^{12}K8$, $pya^{12}K8$ and $thi^{12}K8$, where pya is 2-pyridylalanine and thi is 2-thienylalanine. Each peptide was combined with JTS-1, in lieu of $YKA(K)_8WK$ (K8) which served as reference for transfection efficiency using 20 μg β-galactosidase complexes with a 1:3:1 charge ratio in RAW264 macrophages. The results are shown in Fig. 6.

All peptides transfected RAW264 cells effectively. Transfection efficiency increases in the order $phe^{12}K8 = Tyr^{12}K8 < Ala^{12}K8 < Thi^{12}K8 < His^{12}K8 < Pya^{12}K8$. From these results, it appears that bulky groups such as Trp, Phe, and Tyr diminish transfection slightly, while groups containing nucleophiles (Thi, His, and Pya) enhance transfection. There were no indications of toxicity. The structure–function relationship of these peptides for DNA binding remains unexplored.

4.3.3.3. *Analogs of GLFEALLELLESLWELLLEA*

The effects of modifications of JTS-1 on the transfection efficiency in C_2C_{12} myotubes were determined using β-galactosidase complexed with

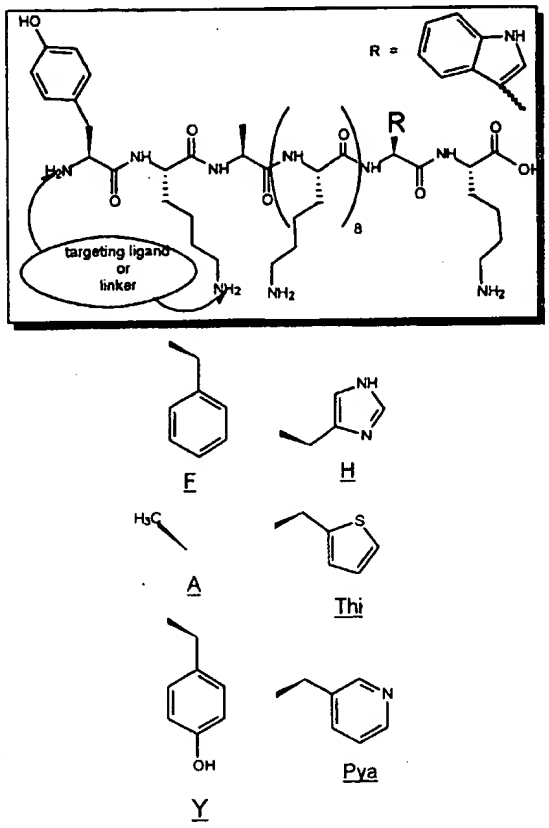


Fig. 5. Analogs of $YKAK_8WK$.

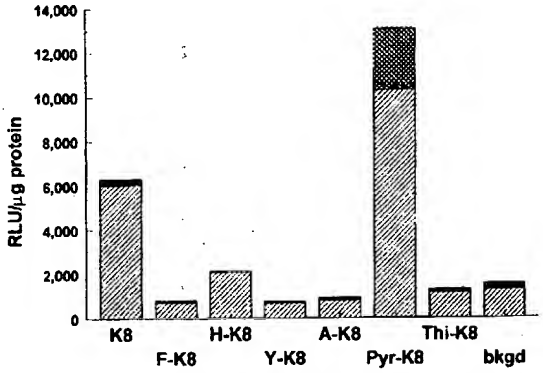
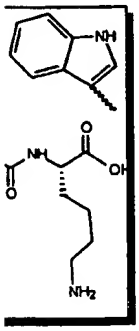


Fig. 6. Gene delivery by analogs of $YKAK_nWK$.

K8 (Duguid, 1996; unpublished data). The amino acid modification are shown in bold type, with JTS-1 as reference.



JTS-1	GLFEALLELLESLWELLLEA
JTS-3	GLFEALLELLEELWELLLEA
JTS-4	GLFEALLELLEELWEALLEA
JTS-6	GLFEALLELLESLWELLLEAGGGGC
JTS-7	SLFEALLELLESLWELLLEA
JTS-8	GLFEALLELLESLYELLLEA
JTS-9	GLFEALAELLESLWEALLEA
JTS-10	GLFEALLELLESPWELLLEA
JTS-11	GLFEALLELLESLWEFLLEA
JTS-12	GLFEAILELLESLWELLLEA
JTS-13	GLFEALLESLWEA
JTS-16	GLFEALLELLESLWEA

High transfection efficiencies were observed for JTS-1, JTS-3 and JTS-8 (Fig. 7). Detectable transfections were also observed for JTS-4, JTS-12, and JTS-16. No transfections were observed for JTS-6, JTS-7, JTS-9, JTS-10, JTS-11, or JTS-13. Most complexes were aggregated near neutral pH. Low level or no transfection was observed if the peptide showed hemolytic activity at both pH 7.4 and 5.0. In addition, JTS-7 and JTS-9, which showed lytic activity at pH 7.4, appeared to be cytotoxic.

Exceptional lytic activity at pH 5 but not at pH 7.4 was predicted by the hemolytic assay for JTS-1, JTS-3, JTS-4 and JTS-8. JTS-1, JTS-3 and JTS-8 showed very high levels of transfection efficiency, while that given by JTS-4 was much lower. Thus, lytic activity of a JTS analog at pH 7.4 results in cell toxicity and low transfection efficiency, while high levels of lytic activity at low peptide concentrations

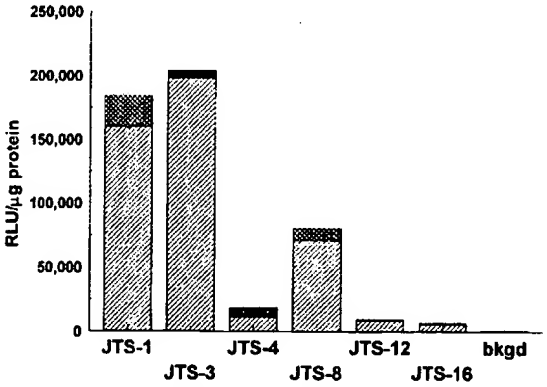


Fig. 7. Gene delivery with analogs of GLFEALLELLESLWELLLEA.

results in a higher transfection efficiency. The prediction from the hemolytic assays is that transfection efficiency would increase in the following order:

JTS − 6, JTS − 7, JTS − 9, JTS − 12, JTS − 13, JTS − 16 < JTS − 10 < JTS − 1, JTS − 3, JTS − 4, JTS − 8.

The experimental results are the following:

JTS − 6, JTS − 7, JTS − 9, JTS − 10, JTS − 11, JTS − 13 < JTS − 12, JTS − 16, JTS − 4 < JTS − 1, JTS − 3, JTS − 8.

With the exception that JTS-10 showed little transfection efficiency, the hemolytic assay appears to be a useful predictor of transfection efficiency in vitro.

5. Antigenic properties of lysine oligopeptides

5.1. Poly(L-lysine) and conjugates of poly(L-lysine)

Early studies found that random linear homopolymers of amino acids were rarely antigenic [76]. McDevitt and Benacerraf note that the antigenicity of these polypeptides has been evaluated in animals after repeated immunizations with complete Freund's adjuvant [77]. The immunogenicity of the polypeptides depends primarily upon the degree of complexity. Nonantigenic homopolymers behave as excellent carriers for haptens, if the polypeptide is long enough. For example, studies of dinitrophenyl–poly(L-lysine) in responder and nonresponder guinea pigs show that α,N-DNP-hexalysine and lower polymers are not immunogenic and are not able to elicit delayed hypersensitivity reactions in animals immunized with the polymers of greater molecular weight [78]. Except in guinea pig strains that possess the poly(L-lysine) gene [79,80], several groups have been unable to elicit antibody formation with either poly(L-lysine) or poly-L-glutamic acid [81–84], although aggregates containing both poly(L-lysine) and poly-L-glutamic acid are immunogenic in rabbits [85]. The ionic aggregates are about 10% as active as the linear polypeptide containing 60% glutamic acid and 40% lysine. The majority of the antibodies elicited by the aggregate are directed against poly(L-

lysine). By contrast, the $glu_{60}lys_{40}$ copolymer does not give an immune response in humans [86] or in mice [87]. The existing literature suggests that the immune response to synthetic DNA delivery systems will be species specific and is likely to give a minimal, if any, response when the DNA-poly(L-lysine) is injected intravenously or into the portal vein, particularly if the complex is formed with hexalysine derivatives. The antigenicity of the aggregated poly(L-lysine) and poly(L-glutamic acid) is a compelling reason to obtain physically homogeneous, chemically defined reagents for gene delivery in vivo. As noted above, Stankovics et al. [11] found that poly(L-lysine) conjugates of ASOR to be antigenic.

5.2. $YKAK_8WK$ and GLFEALLELLESLWELLLEA

The objective was to determine whether or not these DNA complexes produce a significant immune response, i.e., sufficiently strongly to preclude their repetitive use in gene delivery, as is the case with adenovirus. Two conditions were compared: one using Freund's adjuvant intraperitoneally which usually gives a strong immune response, and the second in a subcutaneous protocol similar to that used for gene therapy. The reagents were JTS-1–GGGC–bovine serum albumin (BSA) conjugate, K8–GSGSGSGSGSC–BSA conjugate, DNA–K8–JTS-1 (1:4:1 charge ratio of phosphate, amino, and carboxyl groups), JTS-1–GGGC–SS–CGGG–JTS-1 aggregate and Freund's adjuvant for the standard immunization protocol. The reagents were K8, JTS-1, DNA, JTS-1–GGGC–BSA conjugate, K8–GSGSGSGSGSC–BSA conjugate, and DNA–K8–JTS-1 (1:4:1 charge ratio) in 250 mM sucrose for the gene therapy protocol. The enzyme-linked immunosorbent assay reagents were JTS-1–GGGC–ovalbumin conjugate, K8–GSGSGSGSGSC–ovalbumin conjugate, DNA–K8–JTS-1 (1:4:1 charge ratio), K8, JTS-1 and DNA. The test animals for each antigen were 4 Balb/c female mice, 5–7 weeks old. After priming, samples were collected at 13, 28, 42, 56, and 90 days, with boosts at 21 and 50 days. $YKAK_8WK$, alone, in a complex, or conjugated, does not give a detectable antigenic response, with or without adjuvant. A weak response was seen for JTS-1, about 1000 times weaker than the titer (10^6) for BSA to which it was conjugated, but only when co-administered with Freund's adjuvant. Consistent responses were seen with the JTS-1–GGGC–BSA conjugate and JTS-1–GGGC–SS–CGGG–JTS-1 aggregate. With DNA–K8–JTS-1, only with Freund's adjuvant, two of four animals gave a detectable immune response. There were no observable effects, either acute or long term, on the health of the animals. We conclude there is little immune response to these DNA formulations and that the observed response is not sufficient to preclude their repetitive use in gene delivery in vivo. It is clear that each set of peptides used for DNA delivery will need to be tested as an antigen, independent of its perceived relationship to other peptides.

6. Conclusions

The long term goal is to construct self assembling DNA complexes using synthetic chemical components that perform the roles of proteins in the viral vectors, but without their limitations. The early studies with poly(L-lysine) conjugates of receptor ligands have clearly demonstrated that receptor-mediated gene delivery provides the means to achieve cell specific delivery of DNA complexes. However, the polydispersity of commercial poly(L-lysine) precludes a molecular definition of the final DNA complexes that are frequently used for gene delivery. Synthetic peptide-based DNA delivery systems allow rational design and systematic evaluation of specific functional motifs. With these molecularly defined reagents, it should be possible to obtain a nonviral DNA delivery system with the following properties: (a) structurally well characterized, nontoxic, biodegradable, and nonantigenic systems that protect DNA from degradation and are stable in biofluids; (b) cellular uptake mediated by cell specific plasma membrane receptors; (c) rapid dependent release from the endosome; (d) efficient dissociation of the DNA from the complex into the cytoplasm for transport of the DNA to the nucleus; (e) controlled duration and magnitude of expression.

Two unique features of synthetic peptide-based DNA delivery systems are (a) the absence of biohazards related to the viral genome as well as the production of the viral vector and (b) the absence of

limitations on the size of the therapeutic genes that can be inserted in the recombinant viral vector. In principle, if the gene can be cloned into an expression plasmid, it can be delivered as a synthetic DNA complex. Since these synthetic delivery systems are composed of small peptides which may be poorly antigenic, they hold the promise of repeated gene administration, a highly desirable feature which will be important for gene targeting in vivo to endothelial cells, monocytes, hepatocytes and tumor cells.

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Obstacles and advances in non-viral g n d livery

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This review focuses on recent progress and novel strategies to improve the efficiency of in vivo non-viral gene delivery. Examples of the most promising attempts to overcome specific barriers are presented in fuller detail. Current research into several of the most difficult steps in the gene delivery pathway is discussed including particle stabilization, targeting, cytoplasmic entry and access to the nucleus. The impact of recent reports on our current understanding of the true limitations to in vivo delivery is also discussed. The importance of preclinical animal models for the development of clinical applications of gene therapy is noted.

Keywords Cytoplasmic entry, gene-delivery, non-viral, particle stabilization, targeting

Introduction

In vivo gene delivery to achieve endogenous protein expression has been approached by numerous avenues including recombinant viruses [1,2], protein-DNA polyplexes [3,4] and lipid-based vehicles [5] among others. These methods have great potential for systemic and regional therapies. Essentially any recombinant protein therapy could be transformed into a gene therapy. In many cases gene therapies would be a considerable improvement over existing therapies because of putative advantages in dosing schedule, patient compliance, toxicity, immunogenicity and cost. For example, consider interferon (IFN)-α therapy for hepatitis. Sustained *in vivo* expression of human IFNα upon delivery of the gene to parenchymal cells of the liver has the potential to significantly enhance the current standard IFN therapy for chronic viral B and C hepatitis. The standard treatment regimen involves minimally 3, and as many as 18 to 24 months of daily or thrice weekly injections of 3 to 5 MU of IFNα [6,7]. Rapid elimination from circulation of the injected protein results in peak serum concentrations of 100 to 600 pg/ml, occurring at 4 to 12 h post-injection and complete elimination within 16 to 24 h [8], thus necessitating frequent dosing. A gene therapy approach to continuously produce IFN *in vivo* for extended periods of time would eliminate the need for frequent treatments. Additionally, transgene-directed synthesis and secretion of IFN within the liver, which is the key site of infection, could offer significant advantages compared to systemic distribution of injected protein. Expression *in situ* may achieve sufficient local concentrations of IFN protein without high extrahepatic accumulation, thus minimizing the well described side effects [9-11] of IFN therapy. Intracellular production may also stimulate additional or increased antiviral mechanisms not normally invoked by extracellular binding of IFN [12].

Unfortunately, to this point, reported non-viral gene delivery methods have given less than satisfactory results when evaluated in preclinical animal models or human clinical trials. Essentially, the disappointment can be ascribed primarily to low levels of protein expression as a consequence of low delivery efficiency. The present situation has led to a focus on increasingly complex delivery systems as investigators try to achieve the delivery efficiency that viral systems already demonstrate. Due to the large capacity of endogenous barriers against foreign materials, it does not appear feasible to improve delivery by overwhelming the system with massive doses of DNA, irrespective of the non-viral delivery method chosen. For example, studies of endosomal escape [13] and nuclear transport [14••] report that only 0.01 to 0.1% of injected particles surpass each barrier. Considering other delivery barriers prior to endosomal uptake, especially in the vasculature, vanishingly small numbers of injected particles achieve the final nuclear destination. The critical and very difficult objective is to create a self-assembling gene delivery system that incorporates similar molecular mechanisms to those that allow viruses to trespass vascular, cellular and intracellular barriers, and effectively deliver viral DNA to the nucleus of mammalian cells. Nevertheless, some modeling of viral delivery is possible. Much progress has been made with regard to production of uniform particles for both polymer and liposomal delivery methods [15•,16,17]. Steric stabilization of materials in vascular compartments has been an area of intense investigation and numerous strategies for surface modification of delivery vehicles have shown positive effects [18-21]. Incorporation of molecular components to accomplish receptor-mediated targeting, endosomal escape and nuclear transport have all been attempted and achieved some success *in vitro* [22••,23]. Translation of that success to *in vivo* systems has not been simple. This is in part due to the increasing complexity of delivery vehicle synthesis with each additional molecular component. Additionally, each molecular component intended to enhance a delivery step has the potential to deleteriously affect some other aspect(s) of gene delivery. For example, endosomal escape components might contribute negatively to plasmid DNA condensation aspects, engender immune system recognition or increase toxicity. Solution-phase interactions of polyplexes *in vitro* are limited to a subset of blood components in tissue culture media and a homogeneous population of target cells. *In vivo*, there is a plethora of molecules and cells that interact with, and possibly inactivate or destroy polyplexes, and as a result, substantially decrease delivery efficiency. Thus, a non-viral delivery system that can fully mimic viral delivery, and still retain its best non-viral characteristics, manufacturability and non-immunogenicity, has yet to be realized. However, recent developments in the arena of polyplex formation and delivery are encouraging.

Stabilization of plasmid delivery vehicl s

Some of the earliest studies examining particle stability were based on DNA degradation assays performed in tissue culture medium containing serum. These reports generally

conclude that DNA complexed with sufficient cationic reagent to create condensed particles are protected from degradation by DNases. The minimum amount of conjugate necessary to provide protection is in agreement with the amount, calculated from the Manning equation, needed to completely bind the DNA, typically a charge ratio of ± 0.9 or greater.

However, *in vivo* stability is not directly predictable from these *in vitro* studies. Blood contains many proteins, lipids, carbohydrates and other molecules that can bind to and destabilize polyplexes. Destabilization can come about by events such as opsonization, salt induced aggregation, decomplexation of components or enzymatic degradation. In addition, within the body, cell surfaces play an extremely important role in interactions with polyplexes. These surfaces include many areas of high negative charge density. The abundance of negative charges on these membranes can far exceed those on the DNA molecule and effectively compete for binding to the cationic conjugates. Thus, cell surfaces can promote dissociation of polyplexes with the ultimate and premature release of plasmid DNA, leading to rapid DNA degradation.

One strategy that has been employed to improve the *in vivo* stability of DNA polyplexes is to covalently crosslink the polyplex surface after particle formation. Trubetskoy *et al* [24] investigated the crosslinking of polyamines in complexes with DNA. They reported that crosslinking of reactive amino groups on the polyplex did provide stabilization against both salt-induced aggregation and dextran sulfate-mediated displacement of DNA. Following crosslinking, neither ζ-potential nor particle size was significantly altered, compared to non-crosslinked PLL/DNA polyplexes. Particle size and ζ-potential for PLL/DNA polyplex at a 6:1 ratio, were found to be 61.6 nm and +59 mV for non-crosslinked versus 62.1 nm and +53 mV for crosslinked polyplex, respectively. This work employed a reducible crosslinking reagent, dimethyl-3,3'-dithiobispropionimidate (DTBP). Unfortunately, the current DTBP crosslinked polyplexes were inactive in gene transfer *in vitro*. The authors speculate that use of a more labile crosslinker could release DNA from polyplexes under normal intracellular reducing conditions. Another group, Kakizawa *et al* [25], has achieved disulfide crosslinking through partial substitution of the amino groups of a pegylated polylysine polymer with a protected sulfhydryl moiety. Deprotection and aerial oxidation after polyplex formation resulted in significant stabilization due to formation of a crosslinked shell. 2 mM Dithiothreitol (DTT) was sufficient to cleave the disulfide linkage. The authors anticipate that intracellular glutathione concentration of approximately 3 mM will be sufficient to release DNA. The glutathione concentrations in blood is in the range of 10 μM and should not decompose the particles. However, no data were reported on the *in vivo* gene expression properties of these stabilized DNA polyplexes.

Blessing *et al* have reported the first clear evidence of monomolecular collapse of plasmid DNA into stable virus-like particles [26••]. This research used a dimerizing cationic detergent to condense DNA. The detergent reagent comprised an alkyl chain of ten carbons with sulfhydryl and guanidinium moieties at one end. In the presence of plasmid DNA, the guanidinium groups bound to phosphate groups and condensed the DNA. As a result of this interaction, sulfhydryl moieties are brought into close proximity on the polyplex surface where they dimerize to form a uniform lipid coating. The results were quite striking. Transmission electron microscopy of an unfiltered solution of the stable oxidized polyplexes showed a very homogeneous population of nearly spherical objects (71% monomers) with a few larger structures. The particle size was 23 ± 4 nm. Using typical DNA and amphiphile molecular dimensions, a rough calculation predicts a minimum sphere of 28 nm diameter when adding the volumes of a 5.5 kb plasmid to that of 5500 lipid molecules. It remains to be determined whether these elegantly formed particles remain intact in the presence of serum, under *in vivo* conditions, or upon exposure to either polyanionic materials or negatively charged cell surfaces.

An alternative crosslinking strategy was employed by Adami *et al* [27] who used glutaraldehyde to crosslink peptide-DNA condensates. Plasmid DNA was condensed with a 20 amino acid peptide, CWK(18), into 70 nm particles. Glutaraldehyde was reacted with ε-amino groups on the particle surface to generate crosslinked peptides through reversible Schiff base formation. At glutaraldehyde-to-peptide ratios of 1 to 4 M equivalents they observed increased resistance to shear stress-induced fragmentation and serum endonucleases. They also reported an 80% decrease in the level of transient gene expression in a cell transfection assay. Finally, in comparison to polyplexes formed with high molecular weight polylysine, the crosslinked peptides afforded less protection and lower transfection efficiency *in vitro*. However, the chemistry used for linking together small peptides on the surface of condensed plasmids is simple and flexible, and may lead to materials with improved *in vivo* performance.

Many groups have reported polyplex stabilization against aggregation and/or interaction with serum components. Kwoh [16] and Banaszczyk [28] synthesized PEG-derivatized polylysine (PL) and demonstrated the ability to condense DNA into particles that did not aggregate in saline. In addition, the pegylated polyplexes demonstrated increased *in vivo* expression in comparison to PL (54-fold) or asialoglycoprotein (ASOR)-PL (4.3-fold) polyplexes.

Targeting

Most of the reports that have described targeted delivery of non-viral DNA polyplexes are based on *in vitro* transfections. The relative paucity of *in vivo* reports is understandable considering the difficulty involved in such mechanistic studies. In reality, one cannot 'prove' any mechanism but rather one must disprove all other possible mechanisms. This is hard enough when considering small molecule organic reactions and becomes an overwhelming task for biological systems. There are just too many possibilities to consider and one is forced to resort to more circumstantial evidence. Delivery or gene expression that can be blocked with excess ligand is suggestive of specific receptor-mediated uptake. However, unlike the simple receptor-ligand systems of classical pharmacology, DNA polyplexes are large, complex, poorly defined macromolecular assemblies that likely interact simultaneously at a variety of interfaces with their environment, particularly in the context of *in vivo* applications.

Interactions of local surface charges may induce binding or repulsion of polyplexes from cells or organs. The size, shape and flexibility of the macromolecular assembly may also be strong factors in the uptake or penetration of polyplexes into specific sites within the body. Typically, large numbers of ligands are randomly incorporated into a polyplex. The abundance and random distribution of receptor-binding sites, as well as the size of the particle likely influence and complicate both binding kinetics and the mechanics of internalization. It is possible that such macromolecules concurrently experience opposing forces such as binding cooperativity among receptors and also competition between simultaneously engaged internalization assemblies. Thus, inhibition of gene transfer or expression by addition of excess ligand molecules may be the result of adventitious binding to polyplex surfaces neutralizing cationic surface charges and reducing non-specific binding and uptake into cells. Conversely, in some systems, particularly *in vivo*, excess ligand may enhance gene uptake and expression by suppression of competition between internalization assemblies, or even by re-routing cell entry from endosomal pathways to others that achieve more direct access to the cytoplasm.

Despite the difficulty of achieving incontrovertible receptor-mediated polyplex delivery *in vivo*, several recent reports describe intriguing results. Erbacher *et al* [29••] added an integrin-binding peptide to polyethylenimine (PEI) and showed 10- to 100-fold increased transfection efficiency of integrin-expressing epithelial and fibroblast cells, even in the presence of serum. Most significantly, the large enhancement factor was lost when aspartic acid was replaced by glutamic acid in the targeting peptide sequence. This is strong evidence that supports the involvement of integrins in this *in vitro* transfection pathway. A more typical approach was reported by Diebold [30] who condensed DNA with mannosylated PEI for targeting to dendritic cells. Uptake of these polyplexes into dendritic cells was inhibited by mannosylated albumin.

A novel galactosylated lysine-serine copolymer was reported to give receptor-mediated gene delivery to the liver. In this study, the authors injected 1.5 ml of polyplex into the tail vein of mice and achieved liver-specific expression [31]. Unfortunately, no control groups were included and large volume injections of even naked DNA produce high levels of gene expression in the livers of mice.

An extremely promising result detailing the successful *in vivo* delivery and expression of transferrin-liposome complexes was recently reported by Xu *et al* [32]. Transferrin-liposome-mediated systemic *p53* gene therapy was used in combination with radiation to treat a human tumor xenograft in a nude mouse model. The authors used multiple treatment controls to demonstrate a functional dependence on the transferrin ligand. Established 50 mm^3 tumors regressed and were completely controlled for 6 months after five treatment doses in combination with radiation during the first few weeks. Radiation alone had no effect. Independent confirmation of the ability to target DNA-liposome polyplexes by transferrin or other ligands would be extremely valuable at this point.

Cytoplasmic ntry

One of the most important attributes of viral-based vectors is their ability to promote destabilization of the host cell membrane to allow the entry of the genetic medicine into the target cell. This process is controlled largely by the coat proteins on the surfaces of viruses. Fujii [33••] has reviewed the effect of electrostatic interactions, hydrophobic forces and structural amphiphilicity on protein-mediated membrane destabilization. He concluded that membrane fusion is mediated primarily by these forces acting in concert with one another. Development of synthetic polymers or other materials mimicking the exquisite molecular interactions of viral coat proteins is an extremely difficult, yet key objective of non-viral delivery systems. Indeed, incorporation of molecular entities into polyplexes to increase membrane penetration has been essayed for many years already. Success with this approach will be a fantastic step towards an artificial virus. Wagner [34] has recently reviewed the field and has noted that ligand-polylysine-mediated gene transfer could be improved by up to 1000-fold with membrane-active compounds *in vitro*. Unfortunately, *in vivo* experimentation has not shown as much promise to date.

Non-synthetic methods to achieve membrane permeabilization leading to cytoplasmic entry are the approaches that have shown the greatest enhancement of gene delivery efficiency. Various methods have been employed in this regard and many have been successful *in vivo*. Most notably, reports from Liu [35•] and Zhang [36•] describe a hydrodynamic effect that leads to a high level of gene expression in the liver after large volume injections into the tail vein of mice. This volume effect, originally reported by Lollo *et al* [37], was shown to result in a 200-fold enhancement of gene expression as injection volumes were increased from 0.5 ml to 1 ml. In these more recent studies, volumes up to 3 ml were administered. Transfection efficiency was shown to be dependent on both volume and rate of injection. For normal 18 to 20 g mice, increasing the injection volume from 0.5 ml to 1.5 ml resulted in a 100,000-fold enhancement of gene expression in the liver. A possible mechanism proposed by Zhang to explain this phenomenon is that the rapid intravenous injection of large volumes causes a transient right-sided congestive heart failure and circulatory back pressure to the liver vessels. This back pressure may function to force injected plasmids past the sinusoidal fenestrations of the liver, through hepatocyte cell membranes and into the cytoplasm. Direct observation of the liver during the procedure indicated that the liver blanched and swelled. Transient increases in liver enzymes indicated some toxicity but Liu reported an identical pattern of expression for repeat administrations spaced 6 days apart. Clearly this is not a process that can be used clinically but it does give an indication of the potential improvement that can be achieved through membrane penetration.

Similar processes using large volumes or high-pressure administration have been used recently to transfect arteries [38] and muscle tissue [39]. These scenarios may be more easily translated into clinical practice. In the first case, the authors demonstrated pressure-mediated non-viral arterial gene transfer by temporarily clamping a section of rabbit carotid artery and administering plasmid DNA under

pressures ranging from 100 to 760 mmHg. In normal rabbit carotid arteries the higher pressure gave a 20-fold enhancement of gene delivery efficiency. The efficiency of pressure-mediated gene delivery was another 6-fold higher in injured atherosclerotic arteries. Increased delivery efficiency in injured tissue was also observed by Rekhter *et al* [40] who applied longitudinal stretching and supraphysiological pressure to segments of rabbit aorta *in vitro* to achieve gene transfer prior to grafting into recipient rabbits. Unstretched grafts exhibited no gene expression.

For pressure-mediated muscle transfection, plasmid DNA was injected into the femoral arteries of rats while blood inflow and outflow were blocked. Significant gene expression was observed in hindlimb muscles and levels were dependent on volume and rate of injection. The vast preponderance of expressing cells were myofibers with very few endothelial cells expressing. These results are similar to a much earlier report that used direct injection into skeletal muscle [41].

Electropermeabilization is another technique that has been used to increase the penetration of genes through membranes. Mir *et al* [42•] used needle electrodes or external plate electrodes to deliver electric pulses to rat and rabbit leg muscles directly following injection of plasmid DNA encoding for fibroblast growth factor. Both methods gave comparable results in various muscle groups, with gene expression ranging from 43 to 3249 ng of luciferase. Results obtained with control groups that did not receive electric pulse stimulation were 2 to 4 logs lower. Another report [43] employed electroporation to increase expression of IL-5 in mouse tibialis anterior muscles. Plasmid DNA was injected and then electrode needles were inserted into the DNA injection site to deliver electric pulses. Five days later serum IL-5 levels were > 20 ng/ml. Mice that did not receive electroporation had serum levels of 0.2 ng/ml. Histochemical analysis showed that electroporation increased both the number of muscle fibers taking up plasmid DNA and the number of plasmids per cell. Other groups have used electric pulse methods to achieve enhanced gene delivery into cardiac tissue [44] and corneal endothelium [45].

Nuclear transport

Macromolecules can transit from the cytoplasm into the nucleus through nuclear pore complexes that create passages through the nuclear envelope. The diameter of the channel for active transport is about 25 nm. Numerous nuclear localization signals (NLS) have been identified [46] and many have been incorporated into gene delivery vehicles. In some cases, remarkable improvements in delivery efficiency have been reported.

One strategy has been to attach an NLS peptide by various means to plasmid DNA. Neves *et al* [47] reported on the coupling of a targeting peptide to plasmid DNA by covalent triple helix formation. The reporter gene was expressed in cells, indicating no loss of the gene expression functionality of the plasmid. However, no increase in expression was observed as a result of the NLS peptide. Branden [48] linked a peptide nucleic acid (PNA) to a NLS to create a bifunctional agent that binds tightly to specific sequences on plasmid DNA. Gene expression was preserved when hybridized to PNA-NLS and the efficacy of plasmid transfection was improved up to 8-fold *in vitro*. The most encouraging result thus far was reported by Zanta *et al* [22]. They synthesized a double stranded linear capped CMV*luciferase-NLS* gene containing a single NLS peptide (PKKKRKVEDPYC) at one terminal end of the DNA molecule. Transfection enhancement of up to 1000-fold was observed in dividing HeLa and 3T3 cells and 10- to 30-fold enhancement was seen in macrophages, neurons and hepatocytes. The enhancement could be abolished by a single lysine to threonine mutation of the NLS peptide. This sequence selectivity suggests involvement of the nuclear import machinery. The methods Zanta *et al* used to create a capped linear DNA molecule with a single NLS attachment site were laborious and low-yielding. The authors were unable to produce sufficient materials to examine its *in vivo* activity. Alternative techniques would have to be employed to make this strategy feasible for eventual clinical studies.

Subramanian [49] used a more conventional method of binding an NLS to plasmid DNA. A cationic peptide scaffold was conjugated with the less commonly used M9 NLS of heterogeneous nuclear ribonucleoprotein A1. Lipofection of confluent endothelium with plasmid complexed to the conjugate resulted in 83% transfection and a 63-fold increase in marker gene expression. Wheat germ agglutinin, a known blocker of nuclear pores, was able to abolish the nuclear uptake. A similar concept was used by Chan [50] who conjugated an NLS from SV40 large tumor antigen to polylysine. This conjugate was cocomplexed with transferrin-polylysine to reporter plasmid. Polyplexes containing the NLS sequence showed a modest increase (1.7-fold) in transfection efficiency.

Two groups have recently reported that simple polycationic polymers can promote nuclear transport of transgenes. Glycerol enhanced transferrinfection of resting confluent primary human fibroblasts, as reported by Zauner [51], was shown to give higher transfection levels than lipofection. Lipofection, but not transferrinfection, showed a preference for transfecting cycling cells wherein the nuclear membrane is dissolved and then reformed. Micro-injection of plasmid DNA/polylysine polyplexes into the cytoplasm of fibroblasts resulted in a higher percentage of expressing cells compared to micro-injection of naked DNA. The two observations suggest that the higher transfection levels observed with transferrinfection in resting cells could be due to enhanced nuclear uptake of DNA promoted by cationic polylysine polymer. Similar micro-injection results were reported by Pollard *et al* [52]. Their results corroborate that PEI and PL, but not cationic lipids, increase gene expression when polyplexes are injected into the cytoplasm. Surprisingly, they also report that cationic lipids, but not cationic polymers, inhibit gene expression when polyplexes are injected into the nucleus.

The impact of DNA sequence on plasmid nuclear import has been studied by both Vacik [53] and Dean [54•]. Inclusion of SV40 enhancer sequences increased nuclear transport of cytoplasmically injected plasmids (as monitored by gene expression levels) by up to 100-fold. The difference in expression level was not seen for nuclear injected plasmids nor after cell division of cytoplasmically injected cells. Cell-specific nuclear transport was also reported for

cytoplasmically injected plasmids containing cell-specific promoter sequences. Cell specificity was not seen for plasmids containing SV40 sequences.

Conclusi ns

Despite significant progress in non-viral gene delivery methods, this research field remains in a distinctly preclinical developmental phase. Recently, there have been remarkable advances made in methods of delivery vehicle constitution and formulation. Very small (20 nm) and reasonably uniform polyplexes have been reproducibly synthesized. This is a major step on the path towards formation of a self-assembling system that may lead to an artificial virus for gene therapies. In order to achieve the potency of a virus, other elements will need to be creatively incorporated into the polyplex. The first thought has been to add moieties to enhance targeting, cytoplasmic entry and nuclear transport. Of course, these artificial virus enhancers must not bring along the usual viral problems of immunogenicity, toxicity and low-yield manufacturing. One truly encouraging aspect is that researchers in the non-viral gene delivery field have been extraordinarily inventive. Newer methods to achieve improved gene delivery have not been restricted to the addition of enhancing moieties. Recent administration protocols have employed pressure-mediated and other physical techniques to improve cytoplasmic entry. A wide variety of plasmid modification techniques have enabled greatly increased nuclear transport. It is expected that this sort of open-minded resourcefulness will continue to bring us closer to non-viral delivery systems worthy of clinical investigation.

At this point, the value of preclinical animal models cannot be underestimated. Current researchers have access to numerous laboratory species for delivery and expression studies, in addition to the usual toxicity studies. Although perfect allometric systems do not exist, the ability to quantitatively assess delivery efficiency and even therapeutic efficacy in various species is an important enabling aspect of this research. Common examples that have been used are the hemophilic dog models for Factors VIII and IX, and severely compromised immunodeficient mice (SCID) for various human proteins. It is expected that many knockout gene therapeutic models will be created in the future, and these will prove very useful for demonstrating therapeutic efficacy before entering into a clinical trial phase.

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Review

Gene delivery with synthetic (non viral) carriers

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Abstract

Non-viral gene delivery involving the use of cationic polymer and cationic lipid based carriers still continues to enjoy a high profile due to the safety advantages offered by these systems when compared with viruses. However, there are still problems associated with the use of these agents, notably their comparatively low efficiency and the inability to target gene expression to the area of pathology. On intravenous administration gene expression is found predominantly in the first capillary bed encountered—the lung endothelium. The clinical use of non-viral gene delivery systems in cystic fibrosis or cancer has involved their direct application to the site of pathology due to the targeting difficulties experienced. For gene expression to occur genes must be transported to the interior of the cell nucleus and a number of biological barriers to effective gene delivery have been identified. These may be divided into extracellular such as the targeting barrier mentioned above and intracellular such as the need for endosomal escape after endocytosis and the inefficient trafficking of genes to the nucleus. Targeting ligands have been used with moderate success to overcome the targeting barrier while endosomal escape and nuclear targeting peptides are some of the strategies, which have been employed to overcome the problems of endosomal escape and nuclear trafficking. It is hoped that the next generation of carriers will incorporate mechanisms to overcome these barriers thus improving the efficacy of such materials. © 2001 Published by Elsevier Science B.V.

Keywords: Non-viral gene delivery; Polymers; Cationic liposomes; Polymeric vesicles; Dendrimers

1. Introduction

The completion of a working draft of the human genome project (McIlwain, 2000) paved the way for a greater understanding of genetic diseases. It is now theoretically possible to treat diseases of genetic origin by administering healthy copies of mutated (disease) genes or promote a protective immune response by administering genes encoding for specific antigens. Currently, however, the greatest hurdle to the actual realisation of these therapies is the development of non-toxic and efficacious delivery systems (Ander-

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son, 1998). Gene expression results when DNA is transported inside the cell nucleus of the target cell and there is still a need for carriers, which perform this feat safely and efficiently.

It is currently possible to obtain local transient transgene expression when naked plasmid DNA is administered to muscle tissue (Wolff et al., 1990). To obtain a systemic effect with the injection of naked DNA is difficult however as the intravenous injection of naked DNA results in low levels of gene expression in all major organs (Liu et al., 1995). There is thus an acute need for a delivery system in situations where widespread gene expression is desired such as in the treatment of metastatic disease. Over 500 gene therapy trials have been completed to date (Journal of Gene Medicine, 2001) with the majority using the more efficient viral vectors such as retroviruses and adenoviruses and with only about 20% of trials reporting the use of non-viral vectors. Viral vectors are more efficient yet plagued by safety concerns (Verma and Somia, 1997) and within the last 2 years the safety issues surrounding the use of viruses have been brought sharply into focus with the death of an 18-year-old and reasonably fit gene therapy trial patient (Marhsall, 2000). Hence despite their comparatively low efficiency when compared with viruses, non-viral vectors continue to attract a great deal of interest due to their advantageous safety profile. Add to the safety issues outlined above, the recent report on the comparative activity of retroviral and non-viral (liposomal) gene transfer in a mouse model which found no survival advantage with the use of retroviruses (Princen et al., 2000) and the case for intensified research into non-viral gene delivery becomes even more compelling.

Unfortunately despite the plethora of activity in various gene delivery laboratories world wide, there are still no licensed gene medicines available for the treatment of patients. This review charts the progress achieved so far in the quest to develop safe and effective non-viral gene delivery systems and highlights the major difficulties still facing the area.

Non-viral gene delivery refers to the use of naked DNA (Wolff et al., 1990), cationic lipids formulated into liposomes and complexed with DNA (lipoplexes) (Song et al., 1997), cationic polymers complexed with DNA (polyplexes) (Ogris et al., 1999), polymeric vesicles complexed with DNA (Brown et al., 2000) or a combination of both cationic lipids and cationic polymers complexed with DNA (lipopolyplexes) (Kircheis et al., 1999; Guo and Lee, 2000) (Fig. 1). There have also been attempts to combine the benefits of viral and non-viral systems into one delivery vehicle (Curiel et al., 1991).

2. Naked DNA

The application of plasmid DNA to skeletal muscle cells results in gene expression (transfection) (Wolff et al., 1990). This muscle transfection ability has led to naked DNA being administered intramuscularly for vaccination purposes, where DNA encoding for an antigen is administered with the objective of developing a protective immune response to the transgene antigenic product (Smith et al., 1998; Valez-Fiarcloth et al., 1999). Efficient transfection levels have also been obtained on direct application of naked DNA to the liver (Hickman et al., 1994; Zhang et al., 1997), solid tumours (Yang and Huang, 1996), the epidermis (Yu et al., 1999) and hair follicles (Yu et al., 1999). In some instances the direct application of a transgene using gene carriers such as cationic liposomes does not enhance (Meyer et al., 1995; Balasubramanian et al., 1996) and actually hinders gene expression (Yang and Huang, 1996; Yu et al., 1999; Cohen et al., 2000). The fact that gene expression levels are sometimes not enhanced by gene carriers when the genes are applied directly to certain tissues is evidence that there is no single optimum strategy for delivering genes. Gene delivery for gene therapy will have to be optimised on a mode of administration and disease basis. This lack of enhancement of activity by carriers is observed even when DNA tissue clearance is inhibited by the use of cationic liposomes (Meyer et al., 1995). The deleterious effects of cationic lipids such as *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulphate (DOTAP) to cells such as erythrocytes (Uchegbu et al., 1998) and macrophages (Filion and Phillips, 1998) may be responsible for this observed lack of activity.

It is clear that the application of naked DNA close to the site of pathology and away from degradative elements such as plasma is thus a viable strategy for gene delivery. However this method is ineffective if DNA dosing to anatomically inaccessible sites (e.g. solid tumours in organs) is desired.

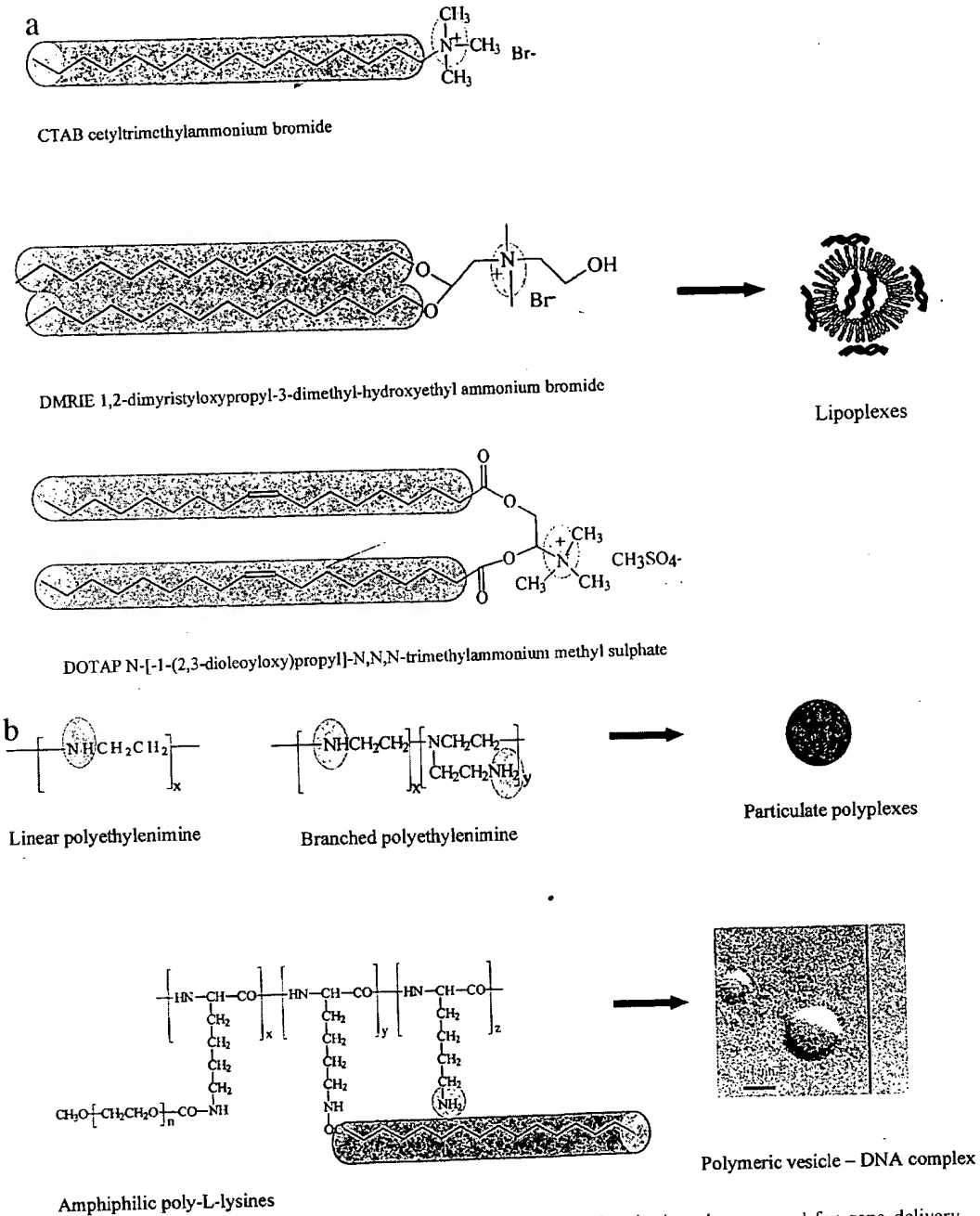


Fig. 1. (a) Examples of cationic lipids used for gene delivery. (b) Examples of cationic polymers used for gene delivery.

3. Cationic lipids

If the administration of genes by the intravenous or oral route or indeed to any remote site is envisaged some form of gene carriage must be employed. Felgner pioneered gene delivery with liposomes formed from lipids with a polar head group (protonated at physiological pH), in 1987 (Felgner et al., 1987). This has since resulted in the commercial production of in vitro gene delivery kits, e.g. lipofectin (*N*-[1-(2,3-di-olyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride, 1,2-dioleoylphosphatidylethanolamine-DOPE, 1:1) and the use of liposomal gene delivery in clinical trials (Porteous et al., 1997; Laitinen et al., 2000). A large number of cationic lipids have been synthesised and studied for gene delivery (Felgner et al., 1994; Remy et al., 1994; Gao and Huang, 1995; Balasubramanian et al., 1996; Budker et al., 1996; Stephan et al., 1996; Lee and Huang, 1997; Rosenzweig et al., 2000; Serikawa et al., 2000; Rosenzweig et al., 2001), some of which are shown in Fig. 1. All cationic lipids possess a hydrophobic group, which may either be one or two fatty acid or alkyl moieties of 12–18 carbons in length or a cholesteryl moiety, in addition to an amine group. The hydrophobic moieties ensure that the cationic lipids assemble into bilayer vesicles on dispersion in aqueous media, effectively shielding the hydrophobic portion of the molecule and exposing the amine head groups to the aqueous medium. The amine group is an absolute necessity for transfection competence as this is the DNA binding moiety interacting electrostatically with DNA and condensing the large anionic molecule into small transportable units—lipoplexes. Structure activity relationship studies have shown that increasing the number of amine groups per molecule (Wheeler et al., 1996) and the distance between the amine groups and the hydrophobic units (Remy et al., 1994) is advantageous to gene delivery. This arrangement of atoms allows an intimate level of DNA binding in the lipoplex (by increasing contact sites) as well as a separation of the bound DNA from the cohesive interaction of the hydrophobic units. Lipoplexes range from 50 nm to just over a micrometer in size (Labatmoleur et al., 1996; Song et al., 1997; Templeton et al., 1997). The influence of lipoplex size on transfection efficacy is contrary to what would be expected and the larger lipoplexes have been reported to improve transfection in vitro (Liu et al., 1997; Templeton et al., 1997; Ross and Hui, 1999; Wells et al., 2000).

A positively charged lipoplex is necessary for cell binding prior to internalisation (da Cruz et al., 2001) by endocytosis. Some neutral lipids such as DOPE (Farhood et al., 1995) and cholesterol (Semple et al., 1996; Hong et al., 1997; Liu et al., 1997) have been incorporated into the cationic lipid bilayer with DOPE said to improve in vitro transfection of some cell lines by facilitating endosomal escape (Farhood et al., 1995). The role of cholesterol however is unclear (Hong et al., 1997; Song et al., 1997).

The use of cationic liposomes to deliver genes increases the level of protein expression obtained on intravenous injection (Liu et al., 1995; Barron et al., 1999a,b). One of the reasons for this is that the complexation of DNA with cationic liposomes prevents DNA degradation in the plasma (Houk et al., 1999; Monck et al., 2000). However these carriers are severely limited in their applicability via the intravenous route as they are rapidly cleared by the plasma and accumulate within the lung tissue (Ishiwata et al., 2000) with protein expression seen primarily in the lung endothelium (Song et al., 1997; Song and Liu, 1998; Barron et al., 1999a,b), the first capillary bed encountered. Protein expression is transient, peaking 4–24 h after dosing and disappearing within a week of dosing (Song et al., 1997). Diverting lipoplexes from the lung can be achieved by incorporating polyethylene glycol (PEG) lipids into the lipoplex, a strategy which increases the circulation time of the lipoplexes and allows protein expression in distal tumours (Anwer et al., 2000a,b). This strategy is reminiscent of that used to divert drug carrying liposomes from the liver and spleen (Blume and Cevc, 1990). In vitro, however a PEG coating decreases uptake and gene transfer (Harvie et al., 2000). An alternative means of increasing transfection to sites out with the lung endothelium is the use of targeting ligands (see Section 9).

Cationic liposomes may also be applied directly to the site of pathology in order to avoid the targeting difficulties encountered when administered intravenously. Access to the alveolar epithelium has been achieved via the intratracheal route of administration, resulting in expression of the α-1-antitrypsin (Canonico et al., 1994), the β-galactosidase reporter (Griesenbach et al., 1998) genes and a reduction in the size of pulmonary tumours on administration of the p53 apoptosis-inducing gene (Zou et al., 2000). However, intratracheal administration is not routinely applicable in the clinic and access to the alveolar epithelium has been successfully sought via the use of aerosols (Stribling et al., 1992). Lipoplexes were effective in preventing degradation of DNA during aerosolisation (Crook et al., 1996).

Successful gene delivery to the eye will have an enormous impact on the treatment of genetic eye diseases. Transfection of the retinal ganglion cells has been observed on instillation of lipoplex eye drops with no inflammation reported (Matsuo et al., 1996).

As cell toxicity has been reported with cationic liposomes (Filion and Phillips, 1998; Uchegbu et al., 1998), research into reducing the toxicity of these carriers is required as once this important goal is achieved, the efficiency of these carriers may improve.

In addition to trying to improve the biological properties of lipidic gene carriers, some studies have focused on improving the stability of lipoplexes employing lyophilisation (Li et al., 2000a,b) with the aid of monosaccharide (Allison et al., 2000), disaccharide (Allison et al., 2000) or PEG lipid (Hong et al., 1997) cryoprotectants.

4. Polymers

As with cationic lipids, polymers bearing groups which are protonated at physiological pH have been employed as gene carriers (Fig. 1). The electrostatic attraction between the cationic charge on the polymer and the negatively charged DNA results in a particulate complex—the polyplex, which is the transfecting unit.

4.1. Poly-L-lysine based polymers

The first polycation to be employed for gene delivery was poly-L-lysine conjugated with asialoorosomucoid for hepatocellular gene targeting (Wu and Wu, 1987). Unlike cationic liposomes much of the early work involving the use of polyplexes utilised ligands to facilitate cellular uptake, e.g. asialoorosomucoid (Wu and Wu, 1987), transferrin (Cotten et al., 1990; Wagner et al., 1990; Curiel et al., 1996), folate (Mislick et al., 1995), monoclonal antibodies (Chen et al., 1994; Schachtschabel et al., 1996; Shimizu et al., 1996) and basic fibroblast growth factor (Sosnowski et al., 1996). The gene transfer activity of poly-L-lysine polyplexes without the use of receptor-mediated strategies is poor (Brown et al., 2000) unless endosomolytic or lysosomotropic agents (e.g. chloroquine) are added (Wadhwa et al., 1997; Pouton et al., 1998). This is an important difference in the biological activity of the amphiphilic cationic lipids and the soluble polymer poly-L-lysine. Cellular uptake of and gene transfer by polyplexes in the presence (Schaffer and Lauffenburger, 1998) or absence (Pouton et al., 1998) of targeting ligands is however still dependant on the presence of a positively charged polyplex (Schaffer and Lauffenburger, 1998), presumably to allow interaction with the negatively charged cell surface and subsequently endosomolytic uptake. Lipidic poly-L-lysines complexed to DNA have been prepared and found to be more efficient in vitro gene delivery agents than cationic liposomes (Zhou et al., 1991; Surovoy et al., 1998), evidence of the advantages offered by the use of more efficient DNA binding amphiphiles. Various other poly-L-lysine copolymers have also been shown to transfer genes into mammalian cells such as those incorporating L-tryptophan (Wadhwa et al., 1997), and graft poly-L-histidine (Benns et al., 2000). The conjugation of histidine to ε-L-lysine residues of poly-L-lysine (Midoux and Monsigny, 1999) resulted in a transfecting polyplex which was more efficient than a poly-L-lysine—chloroquine mixture. This graft copolymer enjoyed an enhancement in activity in the absence of chloroquine because of the additional endosomal buffering capacity offered by histidine

which is protonated below pH 6 (Midoux and Monsigny, 1999). In a similar strategy gluconylated polyhistidine has also been used to transfer genes to mammalian cells and again does not require chloroquine to be active (Pack et al., 2000). Hence the use of histidine residues seems to offer an endosomal escape facility.

A further interesting method of preparing poly-L-lysine based polyplexes involves the replacement of some L-lysine residues with cysteine and tryptophan residues (McKenzie et al., 2000). Cross linking of the cysteine residues in the polyplex increased the gene transfer activity of the polyplex, indicating that DNA release may be triggered by the intracellular reduction of disulphide bonds (McKenzie et al., 2000).

Although poly-L-lysine polyplexes prevent the degradation of DNA by serum nucleases (Chiou et al., 1994) in a similar manner to liposomes (Houk et al., 1999; Monck et al., 2000); on intravenous injection, these polyplexes, are bound by plasma proteins and rapidly cleared from the plasma (Dash et al., 1999) again like cationic liposomes (Ishiwata et al., 2000). Polyplex opsonisation by plasma proteins may be suppressed by coating the polyplexes with a hydrophilic polymer such as hydroxypropyl methacrylic acid, and the cellular uptake of the polyplexes may once again be promoted by the conjugation of targeting ligands such as transferrin (Dash et al., 2000) or fibroplast growth factor (Fisher et al., 2000) to the surface of the coated polyplexes.

4.2. Polyethylenimine

Recently both branched (Boussif et al., 1995, 1996) and linear (Ferrari et al., 1997; Chemin et al., 1998) polyethylenimine have been introduced as cationic polymers for gene delivery and unlike poly-L-lysine this polymer shows efficient gene transfer without the need for endosomolytic or lysosomotropic agents or indeed any agents facilitating receptor mediated uptake. PEI is endocytosed by cells and is also believed to facilitate endosomal escape (Klemm et al., 1998; Kichler et al., 2001). As with all the other non-viral gene delivery systems mentioned above a positively charged (Boussif et al., 1995) polyplex is necessary to allow gene transfer to take place. The influence of molecular weight on the activity of PEI is as yet unclear with some reports detailing an increase in gene transfer activity with a decrease in molecular weight (from 100 to 11.9 kDa) (Fischer et al., 1999) and some reports detailing a decrease in activity on decreasing the molecular weight (from 70 to 1.8 kDa) (Godbey et al., 1999). It is likely that an optimum molecular weight exists somewhere between 11.9 and 70 kDa.

PEI is quite an efficient gene transfer agent (Ferrari et al., 1997), however, the addition of targeting ligands to this polymer enhances its activity in some cell lines (Kircheis et al., 1997; Zanta et al., 1997; Erbacher et al., 1999; Li et al., 2000a,b). Recently hydrophobised PEI has been incorporated within DOPE, egg phosphatidylcholine and dipalmitoyl phosphatidylcholine liposomes, producing an efficient gene transfer agent although the activity of this soluble amphiphilic polymer was diminished when administered without the liposomal lipids (Yamazaki et al., 2000). Unfortunately, PEI as with some of the cationic lipids (Filion and Phillips, 1998) has also been reported to be toxic to cells (Boussif et al., 1995; Ferrari et al., 1997; Godbey et al., 2001).

PEI polyplexes have been used to achieve gene expression in experimental animals by direct application to various anatomical sites such as rat kidneys by intrarterial injection (Boletta et al., 1997), mouse brains (Boussif et al., 1995; Lemkine et al., 1999), and mouse tumours (Coll et al., 1999; Aoki et al., 2001) by direct injection and rabbit lungs by intratracheal administration (Ferrari et al., 1997, 1999). PEI gene expression also appears to be transient and is undetectable 14 days after administration to the kidney (Boletta et al., 1997). The direct administration of these polyplexes to organs in order to achieve transduction is evidence of the lack of targeting ability of these polyplexes on intravenous administration.

On intravenous injection of PEI polyplexes, transfection occurs primarily in the lung (Goula et al., 1998; Bragonzi et al., 1999) as with cationic liposomes (Song et al., 1997) with some expression being detected unusually in the alveolar epithelium (Goula et al., 1998). Intravenously administered PEI-transgene is reported to cross

the endothelial barrier and become expressed in alveolar epithelial cells within 2 h after administration (Goula et al., 1998). To divert transgene expression from the lung tissue, once again a PEG coating has been used, resulting in increased transfection in the liver (Nguyen et al., 2000). Additionally the combination of a PEG coating and the transferrin targeting ligand, resulted in gene expression in distal tumour sites (Ogris et al., 1999). PEG not only allows gene transport to sites out with the lung but also modulates the toxicity of PEI (Ogris et al., 1999), although in vitro uptake and transfer is diminished by PEG (Choi et al., 2001) as was observed for cationic liposomes (Harvie et al., 2000). Overall the gene expression seen with linear PEI is superior to that seen with cationic liposomes both on intravenous (Bragonzi et al., 1999) and intratracheal (Ferrari et al., 1997) administration.

4.3. Other polymers

A transfecting peptide has been prepared from the N-terminal of the human adenovirus fibre protein which promotes transport of DNA to the nucleus and shows improved transfection rates when compared with the cationic liposomes prepared from DOTAP (Zhang et al., 1999). Poly(dimethylamino)ethylmethacrylate (Lim et al., 2000; Hennink et al., 2001), poly(*N*-ethyl-4-vinylpyridinium) polymers (Kabanov et al., 1993), poly-L-histidine (Pack et al., 2000) polylactide co-glycolide (PLG) (Cohen et al., 2000) and chitosan (Murata et al., 1996, 1997; Erbacher et al., 1998; Roy et al., 1999) have also been used for gene delivery. Both chitosan (Erbacher et al., 1998) and PLG (Cohen et al., 2000) nanoparticles appear to control the release of DNA and prolong its action both in vitro (Erbacher et al., 1998) an in vivo (Cohen et al., 2000). The use of PLG is an unusual example of the efficacy of a polymer which is not protonated and hence not cationic at physiological pH as the PLG particles actually possess a negative surface charge (Cohen et al., 2000). Chitosan has also been used via the oral route to achieve an immune response against a peanut allergen (Roy et al., 1999), the first report of an orally active gene delivery system.

4.4. Dendrimers

A range of polyamidoamine (Bielinska et al., 1995a,b; Kukowska-Latallo et al., 1996; Bielinska et al., 1997; Du et al., 1998; BenMamoun et al., 1999; Bielinska et al., 1999; Hudde et al., 1999; Toth et al., 1999; Bielinska et al., 2000) and phosphorous containing (Loup et al., 1999) dendrimers have been studied as gene delivery systems. Terminal amino groups bind DNA by electrostatic means (Bielinska et al., 1999) and once again positively charged complexes must be formed and are necessary for gene transfer (Shah et al., 2000). An increase in the level of terminal amino groups appears to enhance gene delivery (Toth et al., 1999). Dendrimer-gene complexes are presumably internalised by endocytosis and there are also advantages associated with the star shape of the polymer as DNA appears to interact with the surface primary amines only, leaving the internal tertiary amines available for the neutralisation of the acid pH (Lee et al., 1996) within the endosomal/lysosomal compartment. Following internalisation, the release of polyamidoamine-gene complexes by the endosome has been attributed to the protonation of the internal tertiary nitrogens by endosomal protons which then results in a swelling of the endosome and the release of the DNA to the cytoplasm (Tang et al., 1996). The hydrolytic degradation of polyamidoamine dendrimer amide bonds in water or ethanol (Tang et al., 1996; Hudde et al., 1999) increases transfection efficacy up to 50-fold which the authors attribute to the increased flexibility of the polymer on heat degradation. This increased flexibility is said to be crucial to the swelling of the endosome (Tang et al., 1996). Partially hydrolysed polyamidoamine dendrimers were found to be more effective gene transfer agents than branched PEI in the in vivo transfection of the carotid artery of rabbits (Turunen et al., 1999), but less effective than branched PEI in transfecting the lungs of mice on intratracheal administration (Rudolph et al., 2000).

5. Polymeric vesicles

Modification of the cationic polymers poly-L-lysine and poly-L-ornithine by the covalent attachment of both hydrophobic (palmitoyl) and hydrophilic (methoxy polyethylene glycol) groups produces amphiphilic compounds capable of forming vesicles in the presence of cholesterol (Fig. 1) (Brown et al., 2000). These amino-acid based systems are less toxic and more efficient at delivering DNA to some live mammalian cells in vitro than the unmodified polymers (Brown et al., 2000). They also do not require lysomotropic agents such as chloroquine to be active (Brown et al., 2000) and this is attributed to the amphiphilicity of these materials.

Polymerised vesicles have been prepared by the self-assembly of polymerisable cationic monomers (Wu et al., 2001). Polymerisation of the liposome reduces the toxicity of the lipoplex without adversely affecting the transfection efficacy of these agents (Wu et al., 2001).

6. Lipopolyplexes

These have been prepared by condensing DNA with a polycation such as poly-L-lysine (Lee and Huang, 1996; Guo and Lee, 2000), polyethylenimine (Guo and Lee, 2000), spermidine (Ibanez et al., 1996) or spermine (Shangguan et al., 2000) and entrapping this polyplex within anionic (Lee and Huang, 1996; Guo and Lee, 2000) or neutral (Ibanez et al., 1996) liposomes. This method of packaging DNA is reported to result in a less toxic (Ibanez et al., 1996; Guo and Lee, 2000) and in some cases more efficient (Ibanez et al., 1996) in vitro gene transfer particle which protects DNA to a greater extent from nuclease degradation (Ibanez et al., 1996; Shangguan et al., 2000) when compared with cationic liposomes alone. It is clear that the removal of the cationic liposome mediated toxicity results in a more biocompatible gene transfer agent. In addition, the incorporation of folate targeting ligands into these lipopolylexes improves gene transfer efficiency (Lee and Huang, 1996).

7. Preparation of non-viral gene delivery systems

Non-viral gene delivery systems are usually prepared by simply mixing a solution of plasmid DNA, encoding for the gene of interest with either the carrier cationic liposomes (Templeton et al., 1997), the carrier polymer (Goula et al., 1998) or the carrier polymeric vesicles (Brown et al., 2000). The resulting particulate system, as indicated above, usually bears a positive surface charge (zeta potential) (Ogris et al., 1999) or a sufficient excess of cationic lipid (Song et al., 1997) to confer a positive surface charge. Particle size is usually in the colloidal size range (Templeton et al., 1997; Brown et al., 2000) and the complexes are administered freshly prepared, as the long-term stability of the complexes has not been proven.

8. The therapeutic use of non-viral gene delivery systems

8.1. Anti-cancer gene therapy

Gene therapy for the treatment of cancer is an area which has great potential once the gene delivery problems specific to these therapies have been adequately addressed (Schätzlein, 2001). Gene therapy of cancer could take the form of the administration of a good copy of a mutated tumour suppressor gene, the administration of a gene encoding for an enzyme which activates an anti-cancer prodrug or the administration of a gene which encodes for an antigen designed to generate a protective immune response (Schätzlein, 2001). A range of proof of concept studies have been carried out in animal models each demonstrating that gene therapy of cancer could one day become a clinical reality. The administration of a liposomal plasmid expressing anti-sense RNA aimed at the suppression of the production of K-ras specific p21 protein has been used to reduce the growth of pancreatic tumours in mice models (Aoki et al., 1995). Also the administration of a liposomal IL-2 gene formulation resulted in tumour suppression in severe combined immunodeficient mice (Egilmez et al., 1996) al-

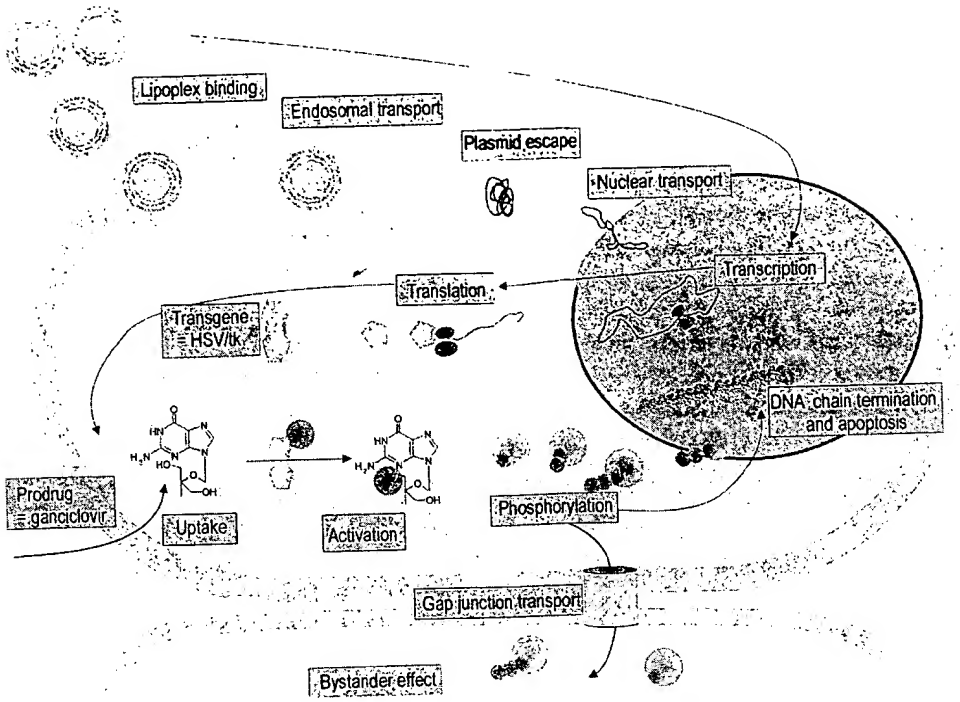


Fig. 2. Schematic representation of the sequence of cellular events occurring after the administration of the herpes simplex thymidine kinase gene for chemotherapeutic activation of the anti-cancer drug ganciclovir.

though the authors conclude that IL-2 independent factors could have resulted in tumour suppression. Other non-viral gene therapy studies with animals which showed good in vivo biological responses include the shrinking of tumours using the gene for the cytokine interleukin 12 (Anwer et al., 2000a,b) or the apoptosis inducing gene p53, which is believed to be defective in certain cancers (Zou et al., 2000).

Viruses are reported to be comparatively more efficacious in transferring genes into mammalian cells both in vitro (Brunner et al., 2000) and in vivo (Verma and Somia, 1997). However, a recent study involving the administration of retroviral and liposomal herpes simplex virus thymidine kinase gene to produce the activating enzyme thymidine kinase for the prodrug ganciclovir (Fig. 2) found no increase in survival in the group of mice administered the retroviral when compared with the animals administered the liposomal formulation (Princen et al., 2000). Ganciclovir is only activated on phosphorylation by thymidine kinase (Connors, 1995) and these studies indicate that the therapeutic use of viral and non-viral systems may not mirror the data obtained either in vitro or even in vivo with reporter genes. As far as possible a specific pharmacological response should be measured with experimental gene delivery systems.

Cancer gene therapy trials have been conducted using non-viral (exclusively liposomal and naked DNA so far reported) gene delivery strategies. The intratumoral injection of a liposomal gene encoding for HLA-B7 in order to generate a therapeutic immune response in melanoma patients (Nabel et al., 1994, 1996; Stopeck et al., 1998) resulted in a partial response (Nabel et al., 1996; Stopeck et al., 1998). Naked DNA has been used in cancer gene therapy trials and hepatocellular carcinoma patients have been injected with p53 in the form of naked DNA and have also shown a partial response (Habib et al., 1996).

8.2. Prenatal gene therapy

The prospect of administering genes in pregnancy in order to prevent the postnatal manifestation of genetic disease was brought closer by the report that the administration of cationic liposomes to pregnant mice also resulted in gene expression in the progeny although this was at a low level (Ochiya et al., 1999). These studies could lead to therapies for the correction of genetic defects in embryos and foetuses although societal ethical concerns may prevent research in this area.

8.3. DNA vaccination

DNA vaccines have been administered intramuscularly (Gregoriadis et al., 1997), intradermally (Braun et al., 1999), intranasally (Klavinskis et al., 1999) and orally (Roy et al., 1999). Cellular and humoral immune responses have been detected with intramuscular injection of naked DNA (Smith et al., 1998) and the use of both cationic lipids (Gregoriadis et al., 1997) and cationic microparticles (Singh et al., 2000) has been shown to enhance this immune response. A naked DNA vaccine encoding a mycobacterial antigen when administered via the intramuscular route effectively protects animals against challenge with mycobacterium avium (Valez-Fiarcloth et al., 1999). As well as the intramuscular route, an epidermal route of DNA vaccine administration is being studied (Braun et al., 1999). Using gold particles, antigen-producing DNA is fired into the epidermis resulting in gene expression in the outer layers of the epidermis (Braun et al., 1999). This method of gene delivery is able to protect animals against challenge by bovine herpesvirus-1 (Braun et al., 1999). Oral gene delivery has been reported recently. The oral administration of chitosan-DNA nanoparticles resulted in a protective immune response in a murine peanut allergy model (Roy et al., 1999). Additionally widespread mucosal immunity has been observed in animals administered intranasal naked DNA, a strategy which could prevent disease transmission over mucosal surfaces (Klavinskis et al., 1999). Patient studies have involved asymptomatic HIV patients being administered an experimental HIV DNA vaccine intramuscularly with patients receiving the highest dose showing an immune response (Boyer et al., 1999). The use of DNA vaccines is predicted to be a growth area.

8.4. Gene therapy of cystic fibrosis

A number of currently incurable diseases such as cystic fibrosis are the result of single gene defects. Gene therapy of cystic fibrosis will involve replacement of the mutated cystic fibrosis transmembrane conductance regulator (CFTR) gene, encoding for the chloride membrane transporter which is defective in cystic fibrosis patients (Porteous and Alton, 1993). One of the earliest reports on the efficacy of gene therapy using a non-viral gene delivery system (lipoplex) was the favourable response obtained with the cystic fibrosis mouse model administered the CFTR gene in a liposomal formulation (Alton et al., 1993). A full restoration of the defective chloride transporter was reported in some animals. Cystic fibrosis clinical studies have used cationic liposomes to deliver the CFTR gene to cystic fibrosis patients and some restoration of a functioning chloride channel has been reported (Porteous and Dorin, 1993; Caplen et al., 1995; Gill et al., 1997). The uptake of lipoplexes is however inhibited in the cystic fibrosis lung by mucus and infective sputum (Alton, 2000).

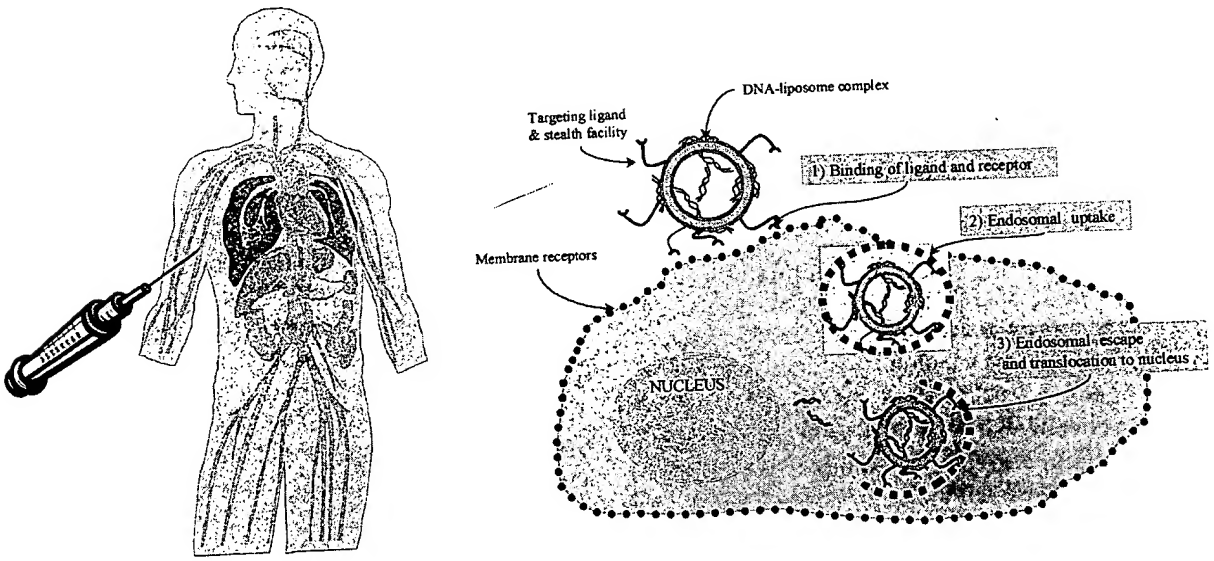
8.5. Other diseases

There are a few other diseases where gene therapy may also have applicability. Arthritis was ameliorated in an arthritic mouse model by the intraperitoneal injection of the interleukin 10 gene, with a decrease in inflammation being observed (Fellowes et al., 2000). Additionally, patients have also been administered the vascular endothelial growth factor complexed with cationic lipids by means of a perfusion-infusion catheter (Laitinen et al., 2000) in an effort to prevent myocardial ischaemia with no adverse effect detected in these preliminary studies although no clinical benefit was also reported (Laitinen et al., 2000).

9. Biological barriers to gene delivery

The ideal gene delivery therapeutic would be an orally active solid dosage form which allowed transport of a large percentage of the dose to the nucleus of the cells within the target tissue. Although oral activity has been documented for a chitosan based formulation (Roy et al., 1999), the above ideal is far from being realised and the current crop of non-viral gene delivery systems are principally most active when administered directly to the site of pathology (Nabel et al., 1996; Stopeck et al., 1998) or as close to the site of pathology as possible (Porteous et al., 1997). The lack of systemic activity on intravenous or even intramuscular delivery and the need to deliver these agents as close to the area of pathology as possible in order to elicit an effect are all evidence of the barriers which have been encountered with gene delivery.

The various barriers to gene delivery have been identified as being both at the extracellular and intracellular level (Schätzlein and Uchegbu, 2001) (Fig. 3) and examples of strategies adopted to overcome these barriers are summarised in Table 1. One of the difficulties faced by the science is the lack of correlation between in vitro and in vivo results (Wells et al., 2000), hence carrier characteristics favouring efficient transfection in vitro may be ineffective in vivo thus making it difficult to identify features which overcome both intracellular and extracellular barriers. Furthermore systematic comparisons of the more widely used non-viral gene delivery systems are not always carried out. A systematic study of both the extracellular and intracellular barriers to gene transfer



EXTRACELLULAR BARRIERS

Degradation of DNA in plasma
Uptake of DNA by reticuloendothelial system
Inability to target DNA to specific organs
Largely ineffective via the oral route – except for immunisation
Transfection inhibited by mucus

INTRACELLULAR BARRIERS

Endosomal escape of DNA
Lysosomal degradation of DNA
Cytoplasmic stability of DNA
Translocation of DNA to the nucleus

Fig. 3. The barriers to non-viral gene delivery.

Table 1
Methods used to overcome extracellular and intracellular barriers

Identified barrier	Strategy employed	Reference
Degradation of DNA by serum nucleases	Complexation with cationic liposomes	Houk et al., 1999
	Complexation with cationic polymers	Chiou et al., 1994
Targeting of DNA to particular tissue types	Targeting ligands, e.g. asialoorosomucoid	Wu and Wu, 1987
	Transferrin	Ogris et al., 1999
DNA uptake by cells	Electroporation	Wells et al., 2000
	Complexation with cationic liposomes	da Cruz et al., 2001
	Complexation with cationic polymers	Kichler et al., 2001
Endosomal escape	Endosomolytic peptides	Lim et al., 2000
	Polyethylenimine	Klemm et al., 1998
Transport from the cytoplasm to the nucleus	Nuclear localisation peptides	Conary et al., 1996
	Nuclear localisation nucleotide sequences	Vacik et al., 1999

encountered by polyplexes, lipoplexes, lipopolyplexes and polymeric vesicles is urgently needed. With the data currently available, it is difficult to compare the ease with which each of the current systems identified above actually overcomes the individual barriers as the usual endpoint in each study is the level of protein expression. Systemic barriers such as the difficulty in targeting specific organs, tissues or cell types as well as the intracellular barriers such as the crossing of the endosomal and nuclear membrane may all pose different levels of challenge to these various systems. Data on any differential activity in this particular context would aid the development of the next generation of more effective and hence clinically useful gene delivery agents.

9.1. Systemic barriers

The intravenous use of lipoplexes (Liu et al., 1995; Barron et al., 1999a,b) and polyplexes (Goula et al., 1998; Bragonzi et al., 1999) increases the level of protein expression obtained on intravenous delivery when compared with levels obtained with naked DNA as these carriers prevent the plasma degradation of DNA (Chiou et al., 1994; Houk et al., 1999) and promote cellular uptake (Labatmoleur et al., 1996; Kichler et al., 2001). However the limitation of using cationic liposomes (Song et al., 1997; Song and Liu, 1998; Song et al., 1998; Barron et al., 1999a,b) and indeed cationic polymers (Goula et al., 1998; Bragonzi et al., 1999) stems from the fact that transfection has been reported to occur primarily in the lung endothelium, the first capillary bed encountered because of the extensive non-specific interaction of the positively charged complexes with elements in the vascular compartment. Reducing the non-specific interactions of liposomes (Anwer et al., 2000a,b) and polymers (Ogris et al., 1999) by incorporating PEG into the lipoplex or polyplex, thus tends to divert the liposomes from the lung and allow transfection of distal solid tumours.

A further method, which has been used to achieve gene targeting, is the attachment of targeting ligands to lipoplexes and polyplexes. As such a variety of targeting ligands have been used to increase uptake in specific cell types, including galactose (Remy et al., 1995; Kawakami et al., 1998, 2000; Nishikawa et al., 2000) or asialorosomucoid (Wu and Wu, 1987; Kao et al., 1996; Singh et al., 2001) moieties for increased uptake by hepatocytes, mannose moieties for targeting the liver macrophages (Kawakami et al., 1998), folate (Lee and Huang, 1996; Guo and Lee, 1999) or transferrin (Ogris et al., 1999; Simoes et al., 1999) ligands for uptake by cells expressing the folate or transferrin receptor and cytoskeleton specific ligands for targeting injured cells from within which the cytoskeleton is exposed on cell injury (Khaw et al., 2000).

Another important barrier recently identified illustrates the importance of studying gene therapy in the context of a specific disease. Gene

transfer to the lung epithelium is severely limited by purulent infective sputum, a normal feature of the cystic fibrosis lung and also by normal mucus (Alton, 2000).

9.2. Cellular barriers

It is quite clear that the delivery of genes by the direct application to cells varies with cell type. Non-viral gene delivery systems are taken up by endocytosis (Farhood et al., 1995; Zabner et al., 1995; Klemm et al., 1998; Kichler et al., 2001) and efforts have focused on effecting the release of these particles from the endosome before the gene is destroyed within this organelle. Uptake may be enhanced by the use of targeting ligands which facilitate receptor-mediated uptake as detailed above and by physical techniques such as ultrasound (Anwer et al., 2000a,b), ionising radiation (Jain and Gewirtz, 1999) and electroporation (Wells et al., 2000). Endosomal escape is said to be facilitated by lipids such as DOPE in some cell lines (Farhood et al., 1995) and also by PEI (Klemm et al., 1998; Kichler et al., 2001).

To improve the gene transfer across both the extracellular and intracellular barrier, a combination of both endosomal disrupting peptides and receptor mediated uptake ligands have been used in lipoplexes and polyplexes. As such the endosome disrupting peptide GALA as well as the targeting ligands transferrin (Simoes et al., 1998, 1999) have been used in cationic liposomal formulations, both the endosomolytic transmembrane domain of diphtheria toxin (Fisher and Wilson, 1997) and asialoorosomucoid have been used with a poly-L-lysine gene delivery system and both galactose and the endosomolytic peptide KALA have been used with poly(2-(dimethylamino)ethyl methacrylate polymers (Lim et al., 2000). All these strategies have enjoyed moderate success. A further variation on the same theme has been the employment of a protein construct with cell targeting ability (in the form of an antibody), an endosomal escape facility (in the form of exotoxin A) and a DNA binding domain (Fominaya and Wels, 1996). All parts of this construct were found to be essential for transfection to occur (Fominaya and Wels, 1996). Finally the conjugation of imidazole units (endosomal escape moieties) to poly-L-lysine improved the transfection activity of this polymer in a dose dependant manner (Putnam et al., 2001).

The use of targeting ligands for receptor mediated uptake and an endosomolytic moiety for endosomal escape has resulted in improvements in gene transfer although the constructs are rather complex entities. The transfer of the gene from the endosome to the cytoplasm once effected then results in the gene encountering a further barrier the nucleolar membrane that is regarded as one of the most significant intracellular barriers to efficient transfection (Zabner et al., 1995; Labat-moleur et al., 1996).

Significant cytoplasmic degradation of un-complexed plasmid DNA can occur within the cytoplasm in hours and could severely limit the total amount of plasmid DNA that can actually be transported into the nucleus (Lechardeur et al., 1999). Transport/access of plasmid DNA to the nucleus occurs during cell division, when the nuclear envelope breaks down, or independently of cell cycling, through pores in the nuclear membrane. While efficient transfection has been shown to depend on mitosis (Brunner et al., 2000) there is also evidence that the nuclear pores act as a size-exclusion barrier. Small DNA fragments (oligonucleotides or plasmids) enter the nucleus by passive diffusion while larger fragments are transported through the nuclear pore complex in an energy-dependent manner (Kreiss et al., 1999; Ludtke et al., 1999). Nuclear pore transport can potentially be improved through the attachment of nuclear localisation signal peptides which redirect intracellular protein transport to the nucleus (Conary et al., 1996; Zanta et al., 1999), or the inclusion in the plasmid of nucleotide sequences with affinity for cellular proteins such as transcription factors; these then mediate the actual nuclear transport (Vacik et al., 1999).

To circumvent the nuclear barrier a cytoplasmic expression system has been developed (Mizuguchi et al., 1997), in which the transgene is administered with T7 RNA polymerase and incorporates a T7 promoter sequence (Mizuguchi et al., 1997). The nucleolar barrier makes the use of RNA expression systems particularly attractive, once

issues surrounding the bulk production and stabilisation of the RNA expression systems have been adequately addressed.

10. Conclusions

The potential market for gene therapeutics must be estimated at billions as opposed to millions of United States dollars. Most of the candidate diseases are currently incurable and some such as cancers are widespread. Non-viral gene delivery has been attempted with liposomal and polymer based systems but unsolved problems remain with these systems. These systems are vulnerable in the plasma, sequestered by particular cell types and on arrival at the cell must be endocytosed, escape from the endosome and eventually deliver DNA to the nucleus. Improvements in all these aspects of gene transport are required if efficient systems are to emerge from the current effort. The cell toxicity associated with the efficient polymeric and liposomal systems hamper their widespread use but principally the main issues surrounding these systems concern their efficiency. The transient gene expression obtained with these systems will be less of a problem if repeat dosing is possible with safe well-tolerated systems.

Results of clinical trials demonstrate that the in vitro and in vivo animal model data can be translated into real clinical benefit and interestingly no major clinical toxicities have been reported with the non-viral delivery systems. It is unlikely that a gene delivery system will emerge which has universal applicability and the first licensed gene therapeutics will utilise a gene delivery system which has been tailored to give high levels of gene expression when administered to treat a specific disease. It is thus more cost effective to concentrate efforts on achieving high levels of therapeutic gene expression in specific well-characterised animal models rather than concentrate excessive effort on work with the ubiquitous reporter genes. The way forward is via multidisciplinary consortia comprising disease experts, chemists and pharmacologists all working together on a specific disease basis. The future of medical technology remains to be revolutionised by these therapies.

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Vectorial delivery of macromolecules into cells using peptide-based vehicles

Jean Gariépy* and Kim Kawamura

The ability to direct the import of therapeutic agents into cells and target them to specific organelles would greatly enhance their functional efficacy. The available spectrum of peptide-based import signals and intracellular routing signals might provide practical solutions towards achieving a guided or vectorial delivery of molecules. Multiple cell-targeting signals and routing domains can be efficiently displayed on branched peptides. These constructs are typically nonimmunogenic in the absence of adjuvant and can be easily assembled using solid phase synthesis. The vectorial delivery of larger complexes, however, will necessitate the development of alternate templates that favor the optimal presentation of all functional signals.

The development of new classes of therapeutic molecules has accelerated dramatically in recent years. This is due to the success of genomic searches in providing an expanding list of cellular targets and also to advances in combinatorial chemistry and high-throughput screening technologies. However, the process of drug discovery goes well beyond the stage of determining lead compounds. Their tissue selectivity and intracellular localization partly define the use of promising therapeutic agents. In future, molecular constructs leading to guided therapies will need to incorporate cellular 'addresses' and intracellular routing signals, as well as the desired functional domains (i.e. an enzyme, prodrug or plasmid). Is it possible to devise molecules that are capable of fulfilling this ensemble of delivery constraints? Presently, the encoding of multiple routing properties into small molecular scaffolds (<1 kDa) is a formidable task.

Currently, two categories of solutions are being evaluated for their ability to address multiple delivery parameters: (1) viral vectors; and (2) nonviral delivery strategies. Viral vectors have been used to deliver genes into eukaryotic cells to regulate cellular functions or to express therapeutically useful proteins. This strategy is still facing major technical hurdles that need to be addressed: issues such as the immunogenicity of the virus, selectivity and tissue tropism, and the regulation and level of gene expression.

Historically, nonviral delivery systems have been less efficient than their viral counterparts in delivering macromolecules into cells. Nonviral delivery techniques include physical approaches (such as microinjection, particle bombardment and electroporation) and chemical approaches (such as cationic lipids, diethyl aminoethyl (DEAE)-dextran, calcium phosphate, proteins, peptides, dendrimers, liposomes and controlled-release polymers) that are mostly aimed at depositing DNA into cells. Unlike viral delivery systems, all these approaches have mainly focused on addressing one major routing parameter, namely to transport therapeutic agents or plasmids across the plasma membrane of eukaryotic cells.

A resurgence of interest in peptide-based vehicles has taken place in recent years following the discovery of short arginine-rich peptide sequences that exhibit efficient intracellular import properties[1–5]. Examples of such import sequences include peptides derived from the HIV Tat protein and the Antennapedia homeodomain (Antp) (Table 1). More importantly, several peptide sequences that alter the routing of molecules inside cells and into organelles have now been identified. Finally, searches principally through phage display peptide libraries have yielded examples of peptides that are able to target cell surface receptors. Thus, of all the nonviral delivery approaches that have been investigated to date, peptide-based vehicles offer the broadest range of options in terms of guiding the delivery of macromolecules to and into cells. Elements from an ever-broadening spectrum of peptide signals could be assembled into structural entities or 'vehicles' that would potentially address multiple delivery constraints. Taken in this context, the term 'vectorial delivery' would simply define the process of delivering a compound or 'cargo' along a distinct routing path that is coded for by one or more localization signals. This review will focus on peptide-based vehicles and how the design of peptide scaffolds might lead to the creation of multi-functional targeted agents for drug delivery.

Localization signals as tools to design guided peptide templates

The specific targeting of tissues or cells with peptides depends on the presence of unique or differentially expressed receptors or markers on cells. Distinct peptide signals can be derived either using structure function-based approaches [i.e. epidermal growth factor (EGF) and related ligands and EGF receptors[6]] or employing combinatorial chemistry or phage display strategies. It is assumed that a growing number of cell-targeting, peptide-based addresses will become

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Table 1. Import signals for crossing the cell membrane[a]

Intracellular import signals	Source	Amino acid sequence[b]	Refs
Hydrophobic sequences			
Membrane Permeable Sequences (MPSs)	Kaposi FGF	AAVALLPAVLLALLAP	25
	Grb2 (SH2 domain)	AAVLLPVLLAAP	23
	Integrin β3	VTVLALGALAGVGVG	24,99
Fusion sequence	HIV-1 gp41 (1–23)	GALFLGFLGAAGSTMGA	26,27
Signal sequence	*Caiiman croc.* Ig(v) light chain	MGLGLHLLVLAAALQGAMGLGLHLLLAAALQGA	27,28
Amphipathic/cationic sequences			
KALA	Influenza HA-2 (1–20)	WEAKLAKALAKALAKHLAKALAKALKACEA	34
GALA		WEAALAEALAEALAEHLAEALAEALEALAA	32
4_6		LARLLARLLARLLRALLRALLRAL	30
Hel 11–7		KLLKLLLKLWKLLLKLLK	30
Penetratin or Antp	Antennapedia third helix (43–58)	RQIKIWFQRRMKKWK	37
Tat	HIV-1 Tat (47–57)	YGRKKRRQRRR	42
VP22	HSV transcription factor (267–300)	DAATATRGRSAASRPTERPRAPARSASRPRRPVE	100
Transportan	Galanin+Mastoparan	GWTLNSAGYLLGKINLKALAALAKKIL	39

[a]Abbreviations: Antp, antennapedia peptide; FGF, fibroblast growth factor; HA, hemagglutinin subunit; HIV, human immunodeficiency virus; Ig, immunoglobulin; SH2, src-homology domain 2; HSV, herpes simplex virus.[b]Amino acid sequence given in single-letter amino acid code.

available for insertion into peptide vehicles. Cell routing elements, however, are presumed to function in most eukaryotic cells. The scope of this review will be limited to a brief survey of notable cell routing signals and their assembly with tissue targeting signals into useful scaffolds. The following will review two dominant classes of routing elements, namely import signals and nuclear localization signals (NLSs).

Import signals

The plasma membrane of eukaryotic cells represents the first barrier that must be traversed by agents acting on intracellular targets. Peptides can cross this barrier either by transient membrane permeabilization approaches, or by endocytosis involving surface interactions, receptors and vesicular compartments. Historically, a broad range of cationic peptides and polymers has been shown to guide the cellular uptake of numerous agents including drugs[7,8] and proteins[9,10], as well as plasmids[11–15]. For example, polylysine, polyarginine and related cationic peptides enter cells efficiently, a property directly related to their mass and the net negative charge on the surface of most eukaryotic cells. Long linear peptide polymers, however, are heterogeneous in length and moderately cytotoxic[16–18]. Over the years, various attempts have been made to increase the efficacy of cellular entry of macromolecules using shorter peptides or chemical moieties. Cellular import has been achieved via receptor-mediated endocytosis. For example, the incorporation of folic acid into anti-T cell receptor (TCR) antibodies allows for folate-receptor targeting on tumor cells that express a high affinity form of this receptor (K_d = 1nM; Refs 19,20) and leads to their endocytosis and eventual T-cell-mediated killing of the cell. The delivery of chemotherapeutic drugs imported through this mechanism is selective, but limited to cells that express folate receptors. Therefore, the emphasis has shifted towards identifying 'generic' peptide-based import signals able to traverse the plasma membrane of most cells. Short peptide sequences capable of directing the movement of a 'cargo' across the cell membrane have now been identified. These sequences function either via endocytotic pathways or through a proposed mechanism referred to as 'inverted micelles'. Based on their amino acid sequence, all known import signals can be broadly classified as either hydrophobic, amphipathic or cationic (Table 1). One group of hydrophobic sequences called membrane permeable sequences (MPSs) was derived from the hydrophobic (h) region of various signal sequences[21] (Table 1). MPSs adopt a characteristic α-helical conformation under membrane mimetic environments, despite the lack of primary sequence homology between signal sequences[22]. These hydrophobic regions can be between 18 and 21 amino acids long. They traverse the cell membrane and are therefore able to import covalently attached functional domains from other intracellular proteins. Examples of such domains include the src homology 2 (SH2) domain of Grb2 (Ref. 23), human integrins β_1, β_3 and α_{IIb} (Ref. 24) and the NLS of NFκB p50 (Ref. 25). Other hydrophobic signal sequences [HIV gp41 fusion peptide, *Caiiman crocodylus* immunoglobulin (v) light chain signal sequence] have also been fused to the NLS sequence derived from the SV40 large T antigen to target the nucleus of cells and deliver antisense oligonucleotides and plasmid DNA (Refs 26–28).

Amphipathic sequences harbor a periodicity of hydrophobic and polar residues. These sequences, typified by the fusion peptide of influenza hemagluttinin (HA–2) (Refs 29–31) and related synthetic analogs [GALA (Refs 32,33), KALA (Ref. 34), 4_6 and Hel 11–7 (Refs 35,36)], represent a

group of import signals that have been shown to interact with cellular membranes. Their interaction with uncharged lipid bilayers results in fusion events with the membrane. The lower pH present in vesicles causes these sequences to undergo a random coil to α-helical transition that induces leakage of vesicular contents. The peptides 4_6 and Hel 11–7 have been shown to transport plasmid DNA into adherent cell lines[35]. However, a high final concentration of peptide (400 μM) was necessary to cause leakage from phospholipid vesicles and the lack of nuclear targeting was most probably the cause of the lower transfection efficiencies observed.

Cationic peptide sequences represent the final group of import signals. As discussed previously, polylysine sequences have been used for several decades as a method of importing various macromolecules across the cell membrane. These sequences interact with the negatively charged phospholipids of the cell membrane and enter the cell via the endocytotic pathway. Penetratin from the third helix of the Antp (Refs 4,37,38) and Transportan created from the fusion of galanin to mastoparan sequences[39], penetrate cell membranes via a postulated 'inverted micelle' pathway. The initial observation that cellular import of Antp occurred at both 4°C and 37°C, ruled out endocytosis as a possible transport mechanism. A translocation pathway was then proposed whereby the cationic component of these peptides allows for their interaction with the cell surface[38] (as found for polylysine). It was then suggested that the accumulation of peptides at the membrane subsequently results in the proposed formation of an inverted micelle that is able to transport the peptide onto the cytoplasmic side. This import mechanism remains speculative. The proposed mode of entry of the basic region from HIV-1 Tat protein into cells probably occurs through adsorptive endocytosis[5,40,41] as opposed to an inverted micelle mechanism[42]. A distinguishing feature of HIV Tat, VP22 and Antp sequences, however, is their high content of arginine residues, which suggests that arginine might confer distinct membrane penetration properties on these peptides compared with lysine-rich cationic sequences. The hydrogen bonding properties of the guanidinium side chain of arginine represents one unique structural feature compared with lysine that might contribute to the efficiency of such peptide signals.

With regards to all peptides listed in Table 1, the effective import of macromolecules into cells typically occurs at peptide concentrations between 1 μM and 200 μM. Most of these peptides have been tested on a limited number of cell lines with the inference that they represent 'generic' import sequences. However, the performance of these import sequences varies considerably from one cell type to another. Thus, there is a need to further delineate and define cellular, as well as peptide sequence parameters affecting their transport properties.

Nucleus-directed signals

In some instances, the tagging of an enzyme or plasmid to a cytosolic peptide import signal might be the only routing task that is required. In general, however, entry into a cell might only represent one of several routing parameters that must be satisfied to create accurately targeted functional agents. For example, an important cellular compartment to reach is the nucleus itself. It is the preferred site of action of DNA intercalators, alkylating agents, as well as protein factors involved in transcriptional regulation. Molecules of <60 kDa can diffuse through nuclear pore complexes and reach this organelle. Moreover, a more directed targeting of this compartment from the cytosol could be achieved using NLS. Although most NLS sequences are bipartite in nature (two short cationic domains separated by several residues), the best known example is the short linear sequence defined by residues 127–133 (KKKRKVE, in single-letter amino acid code) of the SV40 large T antigen[43,44]. Coupling this NLS sequence to proteins has shown to ferry the resulting constructs across the nuclear pore complex and into the nucleus[45–50]. Recently, it has been proposed that the HIV Tat peptide also represents a NLS domain that is recognized directly by importin β rather than by importin α (Ref. 51). Thus, the HIV Tat peptide (residues 47–57) might perform two routing functions by first delivering its cargo to the cytosol en route to the nucleus. The non-classic NLS sequence termed M9, derived from the nuclear ribonucleoprotein A1 (GNQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY), has also been shown to effectively route a plasmid to the nucleus of cells[49,52]. Furthermore, the M9 sequence confers nuclear import and export properties to molecules linked to or carrying this sequence via its interaction with the nuclear shuttle protein, transportin. Thus, at least three distinct classes of nucleus import signals are available to relocate conjugates to this compartment.

Peptide-based scaffolds

In designing peptide vehicles that integrate multiple targeting and routing signals, one is rapidly faced with synthesis constraints, as well as domain presentation issues associated with linear assemblies. In addition, multivalency has now become a dominant feature of recent delivery strategies based on peptides[53–57], protein conjugates[58–60] and dendrimers[61–63]. Structural templates that harbor multiple copies of functional domains typically result in constructs with high binding avidity and enhanced functional activity. Protein toxins such as Shiga toxin, Shiga-like toxins, cholera toxin and the *Escherichia coli* heat-labile toxins best exemplify the concept of multivalency. These proteins are structurally related and are composed of a distinct catalytic A subunit and a pentameric arrangement of identical small receptor binding subunits[64–67]. These pentamers can behave as

lectins; they can bind avidly to glycolipids and gangliosides (GM_1, Gb_3 and Gb_4), thereby prompting the clustering of lipid receptors and the receptor-mediated endocytosis of the toxin. From the perspective of synthetic peptide designs, constructs displaying multiple copies of a short peptide derived from the B1 chain of laminin (YIGSR) have been shown to act as potent inhibitors of tumor growth and metastasis, in comparison to their monovalent peptide counterpart[68]. Similarly, the efficiency of nuclear import for protein conjugates that harbor the SV40 large T antigen NLS has been shown to vary as a function of the number of NLS copies coupled to each conjugate[69,70]. Thus, the multivalent presentation of peptide signals offers a practical solution for enhancing their functional properties.

Design constraints in building multifunctional peptide vehicles

Synthetic conjugates containing multiple peptide delivery signals have been most commonly assembled on either polylysine[71,72], dextran[73–75] or other scaffolds[76,77]. The coupling chemistries used to introduce peptides or other functional moieties into such constructs are often performed in solution, and target amino, hydroxyl or thiol groups on the scaffold. The chemistry of these reactive sites, the broad range of reactivities that they display in the context of peptide signals, and the scaffold itself, frequently result in very large heterogeneous conjugates that are often defined in terms of an average number of components. Consequently, the preparation, mass and heterogeneity of such conjugates present practical challenges (cost, characterization, production and immunogenicity) in terms of their development as viable therapeutic agents. Alternatively, fusion proteins have been made by simply tagging a routing sequence to the functional protein of interest[78–80]. To date, recombinant constructs typically present a linear arrangement of signals, which limits the number of ways multiple signals can be displayed. This design strategy implies that most signals, with the exception of presentations at the N- and C-termini, would be located or embedded within the folded structure of a fusion protein and thus might be less accessible. In addition, the tagging of 'foreign' sequences to proteins of mass >5 kDa might render the overall constructs immunogenic[81], an important consideration when contemplating their use as therapeutic agents. In summary, the size and arrangement of signals on common peptide-based vehicles represent structural limitations that need to be addressed if the vectorial delivery of a molecule necessitates multiple recognition and routing signals. The rational design of peptide scaffolds has therefore become an issue.

Branched peptides

A simple and rapid approach for introducing multiple copies of short peptides during solid phase synthesis was developed more than a decade ago[82,83], to synthesize multiple antigenic peptides (MAPs). MAPs are branched peptides assembled on a short lysine-based scaffold. The rationale for making MAPs was to avoid the use of poorly defined carrier proteins, such as keyhole limpet hemocyanin, when preparing immunogens for the production of peptide-specific antisera in animals. The concept of branched peptides has recently been exploited to create intracellular delivery vehicles incorporating pentalysine motifs and NLSs. These nucleus-directed vehicles are referred to as loligomers, a term derived from the fusion of the Latin root 'loligo' associated with members of the squid family and 'oligomer', a suffix defining them as assemblies of amino acids. Loligomers serve as multi-tasking, peptide-based shuttles that are capable of penetrating cells and self-localizing within cellular compartments[53]. Each branch of a loligomer carries peptide signals that guide their import and localization into cells (Fig. 1). Microscopy and flow cytometry performed on several cell lines have confirmed the vectorial delivery of nucleus-directed loligomers into cells[54]. The proposed mechanism of cell entry and subsequent routing of a nucleus-directed loligomer is illustrated in Fig. 2. Examples of the potential application of loligomers to act as delivery vehicles for 'cargo', such as cytotoxic groups, peptides or macromolecules, have also been reported recently. The observed uptake by loligomers of large molecular entities, such as plasmids, was demonstrated using vectors bearing reporter genes, which suggests that these constructs can act as nonviral transfection agents[55]. The photodynamic probe chlorin e_6, a low-molecular weight agent, was also introduced into a nucleus-directed loligomer during synthesis, resulting in a molecule that was found to be 40–400-fold more potent on a molar basis as a light-activated cytotoxic substrate than the

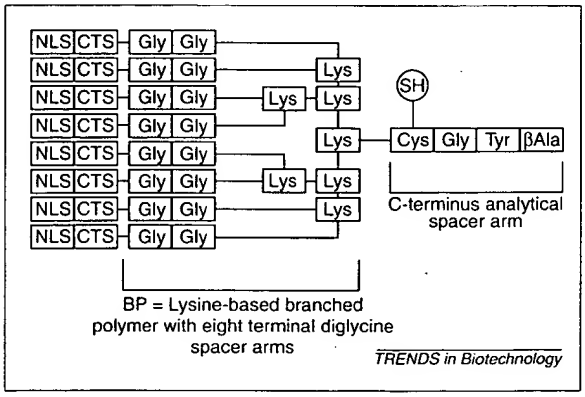

Fig. 1. Structure of loligomer 4, a nucleus-directed branched peptide composed of eight identical N-terminal arms. Diagnostic probes are introduced at the C-terminal analytical arm of the construct via a free thiol group. Peptide branching is carried out during solid phase peptide synthesis by successive additions of di-Boc- or di-Fmoc- protected lysine residues[53,83]. The arms of loligomer 4 consist of a pentalysine sequence acting as a cytoplasmic translocation signal (CTS) and a nuclear localization signal (NLS) from the SV40 large T antigen to direct the construct to the cell nucleus.

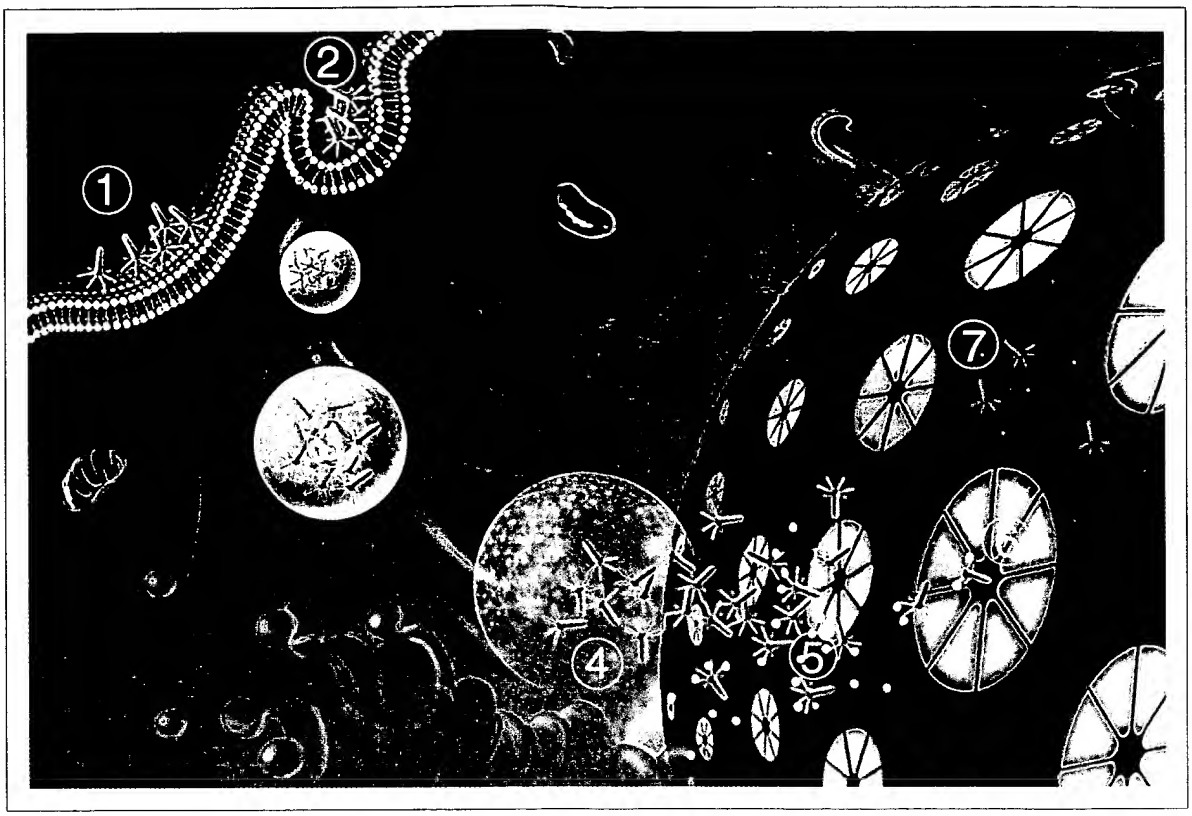

Fig. 2. An artistic representation depicting the import and routing of nucleus-directed loligomers into an eukaryotic cell. Loligomer 4 constructs (tentacular structures) initially bind to membrane surface components and are taken up into vesicular compartments by absorptive endocytosis (stages 1–3). A fraction of the compartmentalized constructs escape to the cytosol (stage 4), where their nuclear localization signals are recognized by cytosolic carriers (stage 5) and the complexes are subsequently imported into the nucleus (stage 6). Loligomers are retained within this compartment (stage 7).

substrate alone[56]. The branched nature of loligomers affords an important advantage over linear peptides in terms of incorporating or positioning multiple copies of tissue-targeting sequences and cytotoxic moieties. Secondly, branching reduces the impact of proteolytic degradation on loligomers because all arms would need to be cleaved to compromise their localization properties. In addition, this scaffold offers an opportunity to locate a diagnostic probe or a functional domain (an antisense oligonucleotide for example) away from the localization signals. Finally, the functional impact of the mass or chemical nature (hydrophobicity) of a reporter group, such as a fluorescent probe or a biotin group, on the localization properties of the resulting construct is less significant in the context of a large branched peptide in comparison to a monovalent peptide. Recent designs of branched peptides for gene delivery[49,84], or modified with a lipid moiety for antigen delivery[85,86], have also highlighted the usefulness and simplicity of assembling such constructs.

Challenges of building multitasking vehicles based on branched peptides

Branched peptides offer advantages over linear peptides in terms of presentation, avidity and stability. Although their masses can exceed 10 kDa, they are not typically immunogenic unless an adjuvant is co-administered with these constructs as part of subcutaneous injections into animals[82]. Interestingly, the coupling of a lipid to MAP peptides results in constructs that are able to raise cytotoxic T cells responses in mice[85], suggesting a selective routing of such lipidated branched peptides into antigen presenting cells. Thus, the integration of non-peptide based routing signals is also possible.

There are, however, some clear limitations in designing branched peptides. Most notably, solid phase synthesis approaches place practical limits on the length or type of functional domains that can be integrated into branched peptide constructs. Functional domains must, therefore, be reduced to a minimal sequence. Even small molecular tags such as human EGF (5 kDa) cannot be easily integrated into branched peptides except through post-solid phase synthesis steps. Off-resin, chemical coupling approaches must be used that are less attractive both in terms of the preparation and of the final construct[83]. Finally, to date, the purification and characterization of loligomers have been hampered by their size and structural arrangement (a consequence of branching). Useful sequential synthesis strategies have now been devised to solve some of these issues and to broaden the use of loligomers[83,87–89]. Alternative platform technologies

might be needed in the future to construct larger and more complex multitasking agents.

Conclusions and future strategies

Designing optimal import sequences

An important conclusion that can be drawn from the currently known peptide-based import sequences (Table 1) is that a finite and relatively small number of parameters define their propensity to enter cells. Thus, the optimization of peptide sequences for cellular delivery is now amenable to random or template-directed combinatorial strategies. For example, a high-throughput screening assay could be devised to search peptide libraries for sequences that are able to noncovalently associate with a plasmid carrying a reporter gene; the resulting DNA–peptide complex is delivered to cells. Theoretically, similar assays could be devised to identify candidate sequences capable of biasing the routing of macromolecules, such as antigens or drugs, to any compartment inside a cell.

Embedding multiple functions into shorter sequences

A detailed understanding of structural features present in short sequences and cellular events defining their routing functions remains sketchy for most peptide signals. Knowledge of these principles is essential in terms of designing minimal peptide-based vehicles. A case in point is the peptide segment that encompasses residues 47–57 of the HIV Tat protein. This 11 amino acid sequence, codes for two functions, namely cellular import[42] and nuclear localization[90], which suggests that multiple functions can be embedded or overlapped within short linear peptide domains. Another striking example can be found in the 30-amino acid tetramerization domain of p53. A leucine-rich nuclear export signal is encoded within its α-helical domain (residues 341–350; human p53). This finding implies that the α-helical segment of p53 serves a crucial structural role in creating the p53 tetramer interface as well as in routing p53 from the nucleus to the cytoplasm[91]. Sequence compression might thus represent a future design strategy where the sequence of functional domains can be overlapped to reduce the dimensions of routing constructs.

Sequence selection and presentation

A cursory analysis of the constructs made to date suggests that the placement of import sequences into peptide constructs varies from one sequence to another and that members of both families of import sequences can be fused to conjugates at either end, resulting in cell penetration. For example, the MPS remain functional when positioned at either terminus of constructs[23]. The HIV Tat import sequence has been typically inserted at the N-terminus of constructs although it normally occupies residues 47–57 of the native 86 amino acid Tat protein[41]. The Antp peptide has been introduced at both the N- and C-terminus of conjugates[92]. Overall, some flexibility in the orientation of import sequences exists within the context of peptide-based vehicles. However, in contemplating their insertion into branched peptides, the nature of the import sequence must be considered given that multiple copies of a hydrophobic import domain linked to a peptide scaffold might alter the overall solubility of the resulting construct. Therefore, cationic or amphipathic sequences are preferable. Lysine-rich regions were initially introduced into loligomers as an import sequence although the basic region of the Tat peptide could theoretically serve the same purpose.

Other routing signals

Macromolecules might also be routed to organelles such as peroxisomes and mitochondria. Targeting signals and pathways leading to the import of proteins into these cellular compartments[93,94] are now being elucidated and should provide some insights into the design of future peptide-based delivery vehicles.

Vectorial delivery based on modular assemblies

As stated previously, the vectorial delivery of peptide constructs would typically follow a path involving a small number of routing tasks. A library of routing elements linked to self-associating elements with the view to creating a broader range of routing vehicles can be envisioned. For example, short peptide sequences coding for coiled coil (or leucine zipper) homodimer and heterodimers could be used as tags inserted in all library elements to allow for the noncovalent pairing of distinct functional domains[95–97]. These modules could be engineered using recombinant approaches, as well as synthetic approaches, thus adding to the complexity of the library. The use of self-associating peptide domains would eliminate the restrictions associated with peptide synthesis and offers the opportunity of building modules using recombinant techniques. This strategy would provide new solutions for the integration of larger peptide or protein units into delivery vehicles. Designed peptide scaffolds such as the four-helix bundle might also represent useful templates for integration of multiple routing signals[98].

In conclusion, peptide scaffolds incorporating drugs and cell localization signals offer novel solutions to the challenge of improving the efficiency of drug delivery to cells. In particular, a polylysine scaffold can be rapidly assembled on a solid phase support and provides a practical starting point for designing delivery vehicles. Alternative assembly scaffolds based on self-associating elements can also be envisioned to introduce more complex targeting signals. Future challenges in designing effective delivery shuttles will lie in identifying, compressing and presenting routing signals into minimal peptide scaffolds.

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Peptide-Mediated Gene Delivery: Influence of Peptide Structure on Gene Expression

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Cationic peptides possessing a single cysteine, tryptophan, and lysine repeat were synthesized to define the minimal peptide length needed to mediate transient gene expression in mammalian cells. The N-terminal cysteine in each peptide was either alkylated or oxidatively dimerized to produce peptides possessing lysine chains of 3, 6, 8, 13, 16, 18, 26, and 36 residues. Each synthetic peptide was studied for its ability to condense plasmid DNA and compared to polylysine$_{19}$ and cationic lipids to establish relative *in vitro* gene transfer efficiency in HepG2 and COS 7 cells. Peptides with lysine repeats of 13 or more bound DNA tightly and produced condensates that decreased in mean diameter from 231 to 53 nm as lysine chain length increased. In contrast, peptides possessing 8 or fewer lysine residues were similar to polylysine$_{19}$, which bound DNA weakly and produced large (0.7–3 μm) DNA condensates. The luciferase expression was elevated 1000-fold after HepG2 cells were transfected with DNA condensates prepared with alkylated Cys-Trp-Lys$_{18}$ (AlkCWK$_{18}$) versus polylysine$_{19}$. The gene transfer efficiencies of AlkCWK$_{18}$ and cationic lipids were equivalent in HepG2 cells but different by 10-fold in COS 7 cells. A 40-fold reduction in particle size and a 1000-fold amplification in transfection efficiency for AlkCWK$_{18}$ DNA condensates relative to polylysine$_{19}$ DNA condensates suggest a contribution from tryptophan that leads to enhanced gene transfer properties for AlkCWK$_{18}$. Tryptophan-containing cationic peptides result in the formation of small DNA condensates that mediate efficient nonspecific gene transfer in mammalian cells. Due to their low toxicity, these peptides may find utility as carriers for nonspecific gene delivery or may be developed further as low molecular weight DNA condensing agents used in targeted gene delivery systems.

Nonviral gene delivery systems are being developed to transfect mammalian host cells with foreign genes (*1–3*). Plasmids encoding transgenes are complexed with carriers that facilitate the transfer of the DNA across the cell membrane for delivery to the nucleus. The efficiency of gene transfer into cells directly influences the resultant gene expression levels. Thereby, optimizing transfer efficiency is often necessary to achieve therapeutically relevant gene expression levels in a variety of host cells (*4, 5*).

Nonviral gene delivery systems rely on carrier molecules to bind and condense DNA into small particles that facilitate DNA entry into cells through endocytosis or pinocytosis (*1*). In addition, the carrier molecules act as scaffolding to which ligands may be attached to achieve site specific targeting of DNA (*6–15*).

The most commonly used DNA condensing agent for the development of nonviral gene delivery systems is polylysine in the size range of dp 90–450 (*6–15*). Its amino groups have been derivatized with asialoorosomucoid, transferrin, carbohydrates, folate, lectins, antibodies, or other proteins to provide specificity in cell recognition, without compromising its binding affinity for DNA (*6–15*). However, the high molecular weight and polydispersity of polylysine also contribute to a lack of chemical control in coupling macromolecular ligands, which leads to heterogeneity in polylysine-based carrier molecules. This can complicate the formulation of DNA carrier complexes and limits the ability to systematically optimize carrier design to achieve maximal efficiency (*16, 17*).

To refine targeted gene delivery carriers aimed at transfecting hepatocytes via the asialolglycoprotein receptor, we previously developed a low molecular weight carrier (4500) by attaching a single complex carbohydrate ligand to low molecular weight polylysine (dp 19) (*18*). This glycopeptide bound to DNA and efficiently transfected HepG2 cells *in vitro* via the asialolglycoprotein receptor, establishing that low molecular weight glycopeptide carriers can function as efficiently as a macromolecular glycoconjugate carriers. However, despite the low molecular weight of this glycopeptide, the polydispersity of polylysine$_{19}$ and the lack of control of the carbohydrate coupling site both contributed to heterogeneity, limiting further opportunity for optimization.

In the present study we have taken the next step toward developing homogeneous glycopeptide carriers by attempting to define the minimal polylysine chain length that leads to DNA condensation. Earlier studies examined the influence of polylysine chain length for transferrin–polylysine-mediated gene delivery and found that the transfection efficiency decreased below dp 300 (*12, 19*). Another study examined the particle size of DNA condensates produced with polylysine varying in size from dp 30 to 1500 and found that low molecular weight polylysine condensed DNA into small particles (20–30 nm) and was also less toxic to cells in culture (*20*). Still others have quantitatively examined the binding of lysine-rich peptides (dp 3–10) to single- and double-stranded oligonucleotides and noted an enhancement in the binding affinity when increasing polylysine chain length up to dp 10 (*21–23*). Notably, the peptides utilized in these studies contained a tryptophan residue that allowed monitoring of DNA binding via fluorescence.

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A recent paper also highlighted the utility of a low molecular weight peptide (dp 13) possessing a lysine repeat of 8 as a DNA condensing for enhancing gene transfer (*24*). When coformulated with a fusogenic peptide and a plasmid encoding luciferase, this peptide mediated gene transfer in several cell lines, including hepatocytes, with efficiency comparable to that of cationic lipid mediated gene delivery.

The synthetic peptides used in the present study also possess a lysine repeat, which was varied from 3 to 36 residues and incorporated one or more tryptophan and cysteine residues. The results establish that a peptide of 13−18 lysine residues possessing a single tryptophan residue enhances gene transfer to cells in culture by up to 3 orders of magnitude relative to comparable polylysine peptides lacking a tryptophan. The mechanism of peptide-mediated gene transfer is related to the efficiency of condensing DNA into small particles. It is proposed that tryptophan plays a specific role in organizing the DNA binding of cationic peptides to produce small condensates that exhibit enhanced gene transfer efficiency. Therefore, tryptophan-containing peptides represent a new class of low molecular weight condensing agents that may be modified with ligands to produce low molecular weight carriers for site specific gene delivery.

MATERIALS AND METHODS

N-terminal Fmoc protected amino acids, and all other reagents for peptide synthesis, were obtained from Advanced ChemTech, Lexington, KY. Minimum essential media (MEM[1]), Sephadex G-25, dithiothreitol, iodoacetamide, iodoacetic acid, and polylysine$_{19}$ (MW 1000−4000) were purchased from Sigma Chemical Co., St. Louis, MO. Ethanedithiol (EDT) was purchased from Aldrich Chemical Co., Milwaukee, WI. Trifluoroacetic acid (TFA) was purchased from Fisher Scientific, Pittsburgh, PA. LB media, LB agar, D-luciferin, and luciferase from *Photinus pyralis* (EC 1.13.12.7) were obtained from Boehringer Mannheim, Indianapolis, IN. HepG2 and COS 7 cells were from the American Type Culture Collection, Rockville, MD. Dulbecco's modified Eagle medium (DMEM), media supplements, and heat inactivated "qualified" fetal bovine serum (FBS) were from Gibco BRL, Grand Island, NY. Bradford reagent was purchased from Bio-Rad, Hercules, CA, and thiazole orange was a gift from Beckton Dickinson Immunocytometry Systems, San Jose, CA. The 5.6 kb plasmid pCMVL encoding the reporter gene luciferase under the control of the cytomegalovirus promoter was a gift from Dr. M. A. Hickman at the University of California, Davis. Peptide purification was performed using a semipreparative (10 μm) C_{18} RP-HPLC column from Vydac, Hesperia, CA. HPLC was performed using a computer-interfaced HPLC and fraction collector from ISCO, Lincoln, NE.

DNA Purification and Peptide Synthesis. Plasmid DNA was prepared by the alkaline lysis method and purified on cesium chloride gradient (*25*). Peptides were prepared by solid phase peptide synthesis on Fmoc-L-Boc-lysine-Wang resin (*p*-benzyloxybenzyl alcohol resin, 1% divinyl benzene cross-linked, 100−200 mesh) at a 136 μmol scale (0.68 mmol/g resin). The synthesis was accomplished using a computer-interfaced Model 90 synthesizer from Advanced Chemtech. Lysine and tryptophan side chains were Boc protected, and the sulfhydryl side chain of cysteine was protected with a trityl group. A 6 molar excess of N-terminal Fmoc-protected amino acid was activated *in situ* in the reaction vessel by adding equimolar diisopropylcarbodiimide and *N*-hydroxybenzotriazole in a total reaction volume of 18 mL. Coupling was carried out for 1 h and was followed with a capping cycle for 30 min with 10% acetic anhydride in 1% diisopropylethylamine. Fmoc deblocking was performed with 25% piperidine for 12 min. All reagents were dissolved in dimethylformamide.

At completion, the resin-conjugated peptide was washed with dichloromethane, dried, and weighed. Cleavage was performed in a solution of TFA/EDT/water (95:2.5:2.5 v/v) for 30 min at room temperature, which simultaneously deprotected the amino acid side chains. The peptide solution was extracted with diethyl ether, concentrated by rotary evaporation, and freeze-dried. Lyophilized crude peptides were dissolved in degassed and nitrogen-purged 0.1% TFA. Peptides (3 μmol per injection) were purified on a semipreparative (2 × 25 cm) C_{18} RP-HPLC column eluted at 10 mL/min with 0.1% TFA and acetonitrile (5−20% over 40 min) while absorbance was monitored at 280 nm, 1.0 AUFS. Purified peptides were concentrated by rotary evaporation, lyophilized, and stored dry at −20 °C.

Lyophilized peptides (1 μmol) were dissolved in 1 mL of nitrogen-purged 50 mM Tris HCl (pH 7.5) and reduced by the addition of 250 μL of 100 mM dithiothreitol prepared in the same buffer by reacting at room temperature for 30 min. Alkylation was carried out by adding 25 mg of solid iodoacetamide or iodoacetic acid followed by reaction for 1 h at room temperature. The alkylated peptides were acidified to pH 2.0 with TFA and purified by RP-HPLC as described above. The yield of each purified peptide (approximately 25%) was determined from the absorbance of tryptophan (ϵ_{280nm} = 5600 M^{-1} cm^{-1}). The TFA salt of polylysine$_{19}$ was prepared by chromatographing the hydrobromide salt on RP-HPLC eluted with 0.1% TFA and acetonitrile while detecting 214 nm as described above. The concentration of polylysine$_{19}$ was established by fluorescamine analysis (*26*) using a calibrated standard of AlkCWK$_{18}$ as a reference.

Dimeric peptides were prepared by dissolving 1 μmol of each purified CWK$_n$ (n = 3, 8, 13, or 18) peptide in Tris HCl (pH 7.5) followed by reaction at 37 °C for 24 h. Each dimeric peptide was purified using RP-HPLC as described above and quantified by Abs_{280nm} (ϵ = 11 200 M^{-1} cm^{-1}).

Peptides were characterized using MALDI-TOF-MS. The peptide (1 nmol) was reconstituted in 100 μL of 0.1% acetic acid, and 1 μL was applied to the target and analyzed using a Vestec-2000 Laser Tec Research laser desorption linear time of flight mass spectrometer using insulin as the internal standard. The instrument was operated with 23 kV ion accelerating voltage and 3 kV multiplier voltage using a 337 nm VSL-SS& ND nitrogen laser with a 3 ns pulse width.

Formulation of Peptide DNA Condensates. Peptide DNA condensates were prepared at a final DNA concentration of 20 μg/mL and at a peptide/DNA stoichiometry varying from 0.1 to 1.5 nmol of peptide/μg of DNA. The condensates were formed by adding peptide (2−30 nmol) prepared in 500 μL of isotonic Hepes-buffered mannitol (HBM, 0.27 M mannitol, 5 mM sodium Hepes, pH 7.5) to 20 μg of DNA in 500 μL of HBM while vortexing, followed by equilibration at room temperature for 30 min.

[1] Abbreviations: RP-HPLC, reversed phase high-performance liquid chromatography; CWK, cysteine−tryptophan−lysine; TFA, trifluoroacetic acid; EDT, ethanedithiol; MALDI-TOF-MS, matrix-assisted time of flight mass spectrometry; RLU, relative light units; DTT, dithiothreitol; FBS, fetal bovine serum; MEM, minimal essential media; DMEM, Dulbecco's modified Eagle media; HBM, Hepes-buffered mannitol; QELS, quasi-elastic light scattering.

Sedimentation of DNA condensates was evaluated by measuring the concentration of DNA in solution before and after centrifugation. After peptide DNA condensates were formed as as described above, a 50 μL aliquot (1 μg of DNA) was diluted in 1 mL of water and the $Abs_{260\ nm}$ was determined on a Beckman DU640 spectrophotometer. Following centrifugation at 13000*g* for 4 min at room temperature, an identical aliquot was diluted with 1 mL of water and the concentration of DNA remaining in solution was determined. The ratio of absorbances subtracted from unity and multiplied by 100 was defined as the percent sedimentation.

Peptide binding to DNA was monitored by a fluorescence titration assay (*18*). A 1 μg aliquot of the peptide DNA condensate prepared as described above was diluted to 1 mL in HBM containing 0.1 μM thiazole orange. The fluorescence of the intercalated dye was measured on an LS50B fluorometer (Perkin-Elmer, U.K.) in a microcuvette by exciting at 500 nm while monitoring emission at 530 nm, with the slits set at 15 and 20 nm and photomultiplier gain set to 700 V. DNA condensation was monitored by measuring total scattered light at 90° by setting both monochromators to 500 nm and decreasing slit widths to 2.5 nm. Fluorescence and scattered light intensity blanks were subtracted from all values before data analysis.

Transmission electron microscopy was preformed by immobilizing condensed DNA on carbon-coated copper grids (3 mm diameter, 400 mesh; Electron Microscopy Sciences, Fort Washington, PA). Grids were glow discharged, and 3 μL of peptide DNA condensate (20 μg/mL), prepared as described above, was placed on the grid for 5 min. The grids were blotted dry and then stained by floating for 1.5 min on each of three 100 μL drops of uranyl acetate (1%, in 95% ethanol) followed by rinsing with 0.4% detergent solution (PhotoFlo, Kodak) and drying. Electron microscopy was performed using a Philips EM-100 transmission electron microscope.

Particle size analysis was measured for peptide DNA condensates prepared at a DNA concentration of 20 μg/mL in HBM and at a stoichiometry of 0.8 or 1.0 ($DiCWK_3$) nmol of peptide/μg of DNA. Samples were analyzed using a Nicomp 370 Autodilute submicrometer particle sizer in the solid particle mode, and acquisition was continued until the fit error was <10. The mean diameter and population distribution were computed from the diffusion coefficient using functions supplied by the instrument.

***In Vitro* Gene Transfection.** HepG2 cells (2×10^6 cells) were plated on 6 × 35 mm wells and grown to 40–70% confluency in MEM supplemented with 10% FBS, penicillin, and streptomycin (10 000 units/mL), sodium pyruvate (100 mM), and L-glutamine (200 mM). Transfections were performed in MEM (2 mL/35 mm well) with 2% FBS, with or without 80 μM chloroquine. Peptide DNA condensates (10 μg of DNA in 0.5 mL of HBM) were added dropwise to triplicate wells. After 5 h of incubation at 37 °C, the medium was replaced with MEM supplemented with 10% FBS.

Luciferase expression was determined at 24 h with some modification of a published method (*27*). Cells were washed twice with ice-cold phosphate-buffered saline (calcium and magnesium free) and then treated with 0.5 mL of ice-cold lysis buffer (25 mM Tris chloride, pH 7.8, 1 mM EDTA, 8 mM magnesium chloride, 1% Triton X-100, 1 mM DTT) for 10 min. The cell lysate mixture was scraped, transferred to 1.5 mL microcentrifuge tubes, and centrifuged for 7 min at 13000*g* at 4 °C to pellet debris.

Lysis buffer (300 μL), sodium ATP (4 μL of a 180 mM solution, pH 7, 4 °C), and cell lysate (100 μL, 4 °C) were combined in a test tube, briefly mixed, and immediately placed in the luminometer. Luciferase relative light units (RLU) were recorded on a Lumat LB 9501 (Berthold Systems, Germany) with 10 s integration after automatic injection of 100 μL of 0.5 mM D-luciferin (prepared fresh in lysis buffer without DTT). The RLU were converted into femtomoles using a standard curve generated each day using luciferase dissolved in Tris acetate, pH 7.5, and stored at −20 °C. The standard curve was constructed by adding a known amount of the enzyme (0.01–100 fmol with specific activity of 2.5 nanounits/fmol) to 35 mm wells containing 40–70% confluent HepG2 or COS 7 cells. The cells were processed as described above, resulting in a standard curve with an average slope of 130 000 RLU/fmol of enzyme.

Protein concentrations were measured by Bradford assay using bovine serum albumin as a standard (*28*). The amount of luciferase recovered in each sample was normalized to milligrams of protein, and the mean and standard deviation obtained from each triplicate are reported.

COS 7 cells were plated at 72 000 cells per well and grown to 50% confluency in DMEM (Gibco BRL) supplemented with penicillin (10 000 units/mL), L-glutamine (200 mM), and 10% FBS for 24 h. The cells were transfected as described for HepG2 cells.

Lipofectace (Gibco BRL, 1:2.5 w/w dimethyl dioctadecylammonium bromide and dioleoylphosphatidylethanolamine) was used to mediate nonspecific gene transfection according to the manufacturer's instructions. The ratio of DNA to Lipofectace was optimized for both COS 7 and HepG2 cells. An optimal DNA/Lipofectace ratio was achieved by dissolving 10 μg of DNA in 100 μL of serum free media (SFM) followed by adding 60 μL of Lipofectace prepared in 140 μL of SFM. The Lipofectace DNA complex was then diluted with 1.7 mL of SFM and used to transfect HepG2 or COS 7 cells for 5 h followed by replacement of the transfecting media with supplemented 10% FBS. The cells were incubated for a total of 24 h, then harvested, and analyzed for luciferase as described above.

Dose response curves were prepared by varying the dose from 1 to 50 μg of DNA while keeping the peptide/DNA stoichiometry fixed at 0.6 nmol/μg of DNA and normalizing the volume to 0.5 mL. Alternatively, a dose response curve for Lipofectace was prepared by varying the DNA dose from 1 to 20 μg while keeping the stoichiometry of Lipofectace to DNA constant and normalizing the total volume of each dose to 2 mL with SFM.

RESULTS

Design of Peptides for Gene Delivery. Cationic peptides were designed to probe the minimal size needed to mediate efficient gene transfer in mammalian cells. The synthetic strategy involved comparison of four peptides with various lysine chain lengths in the range of 3–18 residues. During peptide synthesis, truncated peptides were capped by N-acetylation and a tryptophan residue was placed near the N terminus to provide a chromophore for identification of full-length sequences during purification. This residue allows quantitation of peptide concentration and is also intended for use in monitoring fluorescence to evaluate peptide binding to DNA as previously described (*21*). In addition, each peptide possessed an N-terminal cysteine residue as a potential ligand attachment site.

The four peptides were alkylated with iodoacetamide to provide $AlkCWK_n$ (where $n = 3$, 8, 13, or 18 residues)

Table 1. Peptides for Gene Delivery

name	sequence	mass (obsd/calcd[a])
$AlkCWK_3$	Alk-S-Cys-Trp-$(Lys)_3$	750.2/750.0
$AlkCWK_8$	Alk-S-Cys-Trp-$(Lys)_8$	1391.1/1390.9
$AlkCWK_{13}$	Alk-S-Cys-Trp-$(Lys)_{13}$	2031.1/2031.8
$AlkCWK_{18}$	Alk-S-Cys-Trp-$(Lys)_{18}$	2672.7/2672.5
$DiCWK_3$	$(Lys)_3$-Trp-Cys-S-S-Cys-Trp-$(Lys)_3$	1382.5/1382.8
$DiCWK_8$	$(Lys)_8$-Trp-Cys-S-S-Cys-Trp-$(Lys)_8$	2664.5/2665.2
$DiCWK_{13}$	$(Lys)_{13}$-Trp-Cys-S-S-Cys-Trp-$(Lys)_{13}$	3946.2/3945.9
$DiCWK_{18}$	$(Lys)_{18}$-Trp-Cys-S-S-Cys-Trp-$(Lys)_{18}$	5227.8/5227.9
$polylysine_{19}$	$(Lys)_{19}$	nd[b]/2435.8

[a] Masses are calculated as the average M + 1 of the free base. [b] The mass of $polylysine_{19}$ was not determined due to polydispersity.

(Table 1). A further extension of this peptide series was accomplished by allowing the cysteine of each monomeric peptide to oxidize, resulting in a panel of homodimeric peptides each possessing two tryptophans and a discontinuous lysine repeat of either 6, 16, 26, or 36 residues in length (Table 1). Each alkylated peptide and dimeric peptide was characterized using MALDI-TOF-MS, which produced a dominant ion corresponding to the anticipated molecular weight of each peptide (Table 1).

Purified $AlkCWK_3$, $AlkCWK_8$, $AlkCWK_{13}$, and $AlkCWK_{18}$ each demonstrated a minor (10%) peak eluting later than the major product on RP-HPLC. On storage in an acid solution, the minor peak increased proportionally to the loss of the major product. The new product was isolated and analyzed by MALDI-TOF-MS, which verified a loss of 17 amu. A byproduct of identical mass loss was formed for each of $AlkCWK_3$, $AlkCWK_8$, $AlkCWK_{13}$, and $AlkCWK_{18}$. We speculate that the new product represents a cyclization of N-terminal amine with the acetamido group attached to cysteine leading to the loss of ammonia. The proposed cyclic byproduct of $AlkCWK_{18}$ was isolated and found to be functionally equivalent to the parent structure in transfection assays. Substitution of iodoacetic acid for iodoacetamide in the alkylation step led to an $AlkCWK_{18}$ peptide that was acid stable and functionally equivalent in formulation and biological assays.

Peptide Binding to Plasmid DNA. Peptides were studied for DNA binding using a dye exclusion assay that has been described previously (*18*). Peptide binding to DNA leads to exclusion of thiazole orange intercalation and a decrease in fluorescence. Titration of $AlkCWK_3$, $AlkCWK_8$, $AlkCWK_{13}$, or $AlkCWK_{18}$ with DNA in the range of 0.1−1.5 nmol of peptide/μg of DNA led to a reduction in fluorescence except for the smallest peptide ($AlkCWK_3$), which failed to exclude the intercalator within the titration range (Figure 1A). An asymptote in the fluorescence decline was observed at a stoichiometry of 0.6, 0.4, or 0.2 nmol of peptide/μg of DNA for $AlkCWK_8$, $AlkCWK_{13}$, or $AlkCWK_{18}$, respectively (Figure 1A). The relative fluorescence intensity at peptide/DNA stoichiometries above the asymptote established that $AlkCWK_{13}$ and $AlkCWK_{18}$ were able to exclude thiazole orange intercalation more efficiently than $AlkCWK_8$.

Dimeric peptides ($DiCWK_n$, n = 8, 13, 18) also possessed high affinity for DNA as evidenced by the stoichiometry of the fluorescence asymptote and the reduction in residual fluorescence, both of which correlated with the number of lysine residues (Figure 1B). Of this series, $DiCWK_3$ possessed weak affinity for DNA and thereby produced an asymptote at a stoichiometry of 1 nmol of peptide/μg of DNA.

In contrast to these results, $polylysine_{19}$ demonstrated a markedly different fluorescence titration curve compared to the alkylated or dimeric peptides of comparable length (Figure 1C). Even though $polylysine_{19}$ has a similar number of lysine residues as $AlkCWK_{18}$, its fluorescence asymptote occurs at a stoichiometry of approximately 0.6 nmol of peptide/μg of DNA. This result suggests that $polylysine_{19}$ binding to DNA is weak relative to $AlkCWK_{18}$.

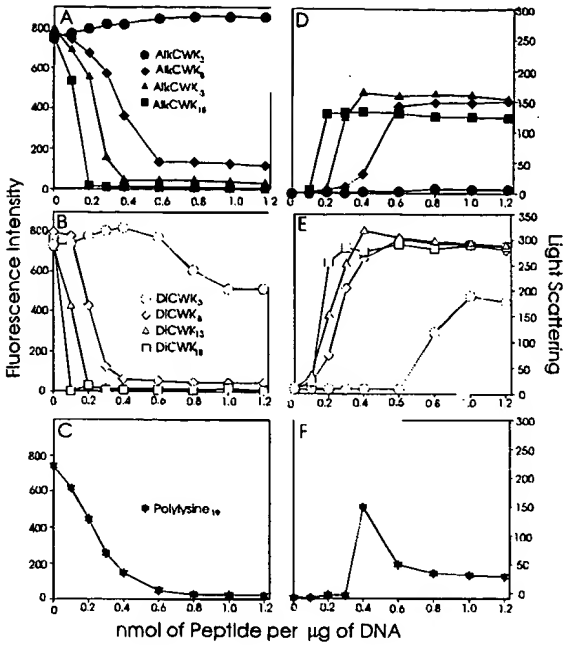

Figure 1. Fluorescence and light scattering titration of peptides with DNA. (A) Thiazole orange fluorescence was determined after titrating $AlkCWK_3$, $AlkCWK_8$, $AlkCWK_{13}$, or $AlkCWK_{18}$ with DNA as described under Materials and Methods. (D) Total light scattering measured simultaneously for each peptide DNA condensate. (B, E) Results of the fluorescence and light scattering titration using $DiCWK_3$, $DiCWK_8$, $DiCWK_{13}$, or $DiCWK_{18}$, respectively. (C, F) Fluorescence and light scattering titration of DNA with $polylysine_{19}$, respectively. Each titration represents the average of three determinations with average standard deviations of 7.4% for the fluorescence titration and 6.2% for the light scattering assay (error bars not shown).

Condensation of DNA with Peptides. Total light scattering at 90° was used to detect the peptide stoichiometry at which condensed DNA particles were formed (*18*, *29*). Titration of $AlkCWK_8$, $AlkCWK_{13}$, or $AlkCWK_{18}$ with DNA produced a maximal total light scattering at stoichiometries that corresponded to the asymptote observed in the fluorescence exclusion assay (Figure 1D). A plateau in the light scattering profile observed at stoichiometries of 0.6, 0.4, and 0.2 for $AlkCWK_8$, $AlkCWK_{13}$, and $AlkCWK_{18}$, respectively, established the complete condensation of DNA at or above this peptide/DNA ratio. In contrast, titration of DNA with $AlkCWK_3$ failed to produce an increase in the light scattering, supporting earlier observations that indicate $AlkCWK_3$ fails to bind to DNA.

Titration of the dimeric peptides with DNA each produced condensates detected by light scattering (Figure 1E). Although the plateau light scattering levels for each dimeric peptide DNA condensate were nearly indistinguishable, the stoichiometry at which the plateau was achieved occurred at 0.6, 0.4, and 0.2 nmol of peptide/μg of DNA for $DiCWK_8$, $DiCWK_{13}$, and $DiCWK_{18}$, respectively. A weaker binding affinity for $DiCWK_3$ was evident from the plateau in light scattering which occurred at a stoichiometry of 1 nmol/μg of DNA (Figure 1E).

The light scattering profile for $polylysine_{19}$ was very distinct from that obtained for alkylated and dimeric

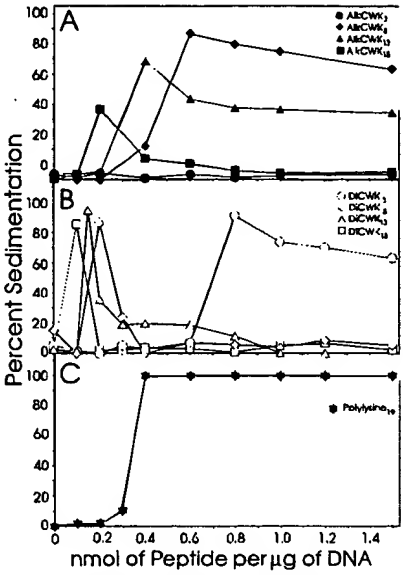

Figure 2. Sedimentation of peptide DNA condensates. The percent of DNA sedimented following centrifugation of peptide-induced DNA condensates is shown. The peptide/DNA stoichiometry was varied from 0.1 to 1.5 nmol of peptide/μg of DNA in HBM at a total DNA concentration of 20 μg/mL. (A) Results for $AlkCWK_3$, $AlkCWK_8$, $AlkCWK_{13}$, and $AlkCWK_{18}$; (B) results for $DiCWK_3$, $DiCWK_8$, $DiCWK_{13}$, and $DiCWK_{18}$; (C) results for $polylysine_{19}$. The average standard deviation for the assay was 8.6% (error bars not shown).

peptides. A sharp increase occurred at a stoichiometry of 0.4 nmol/μg of DNA, which declined to approximately 50 light scattering units at higher peptide/DNA stoichiometries (Figure 1F). This light scattering titration profile distinguished the condensation properties of $polylysine_{19}$ from CWK_n peptides, suggesting differences in the particle size for $polylysine_{19}$ DNA condensates.

Sedimentation of DNA Condensates. To evaluate the relative particle size of DNA condensates prepared at stoichiometries ranging from 0.1 to 1.5 nmol of peptide/μg of DNA, a sedimentation assay was utilized to measure the DNA remaining in suspension following centrifugation at 13000*g* for 4 min (*18*) (Figure 2). Titration of DNA with $AlkCWK_3$ resulted in the complete recovery of the DNA following centrifugation, supporting earlier findings that indicate $AlkCWK_3$ fails to bind and condense DNA into particles. Alternatively, $AlkCWK_8$, $AlkCWK_{13}$, and $AlkCWK_{18}$ each produced maximal sedimentation at a stoichiometry that roughly correlates with the stoichiometry calculated for a charge neutral complex (Figure 2A). At stoichiometries greater than charge neutral, $AlkCWK_8$ condensates sedimented to a greater extent than $AlkCWK_{13}$ or $AlkCWK_{18}$ condensates, indicating their larger size.

A similar trend was observed when dimeric peptide DNA condensates were sedimented. The maximal sedimentation was observed at a stoichiometry of 0.8, 0.2, 0.15, and 0.1 nmol of peptide/μg of DNA for $DiCWK_3$, $DiCWK_8$, $DiCWK_{13}$, and $DiCWK_{18}$, respectively (Figure 2B). At stoichiometries above the calculated charge neutral point $DiCWK_8$, $DiCWK_{13}$, and $DiCWK_{18}$ DNA condensates failed to sediment, suggesting they are smaller in size (Figure 2B). It is also evident that $DiCWK_3$ DNA condensates were large due to the observed sedimentation (70–80%) at stoichiometries above the charge neutralization point (Figure 2B).

In contrast, $polylysine_{19}$ DNA condensates sedimented completely at 0.2 nmol of peptide/μg of DNA and failed to recover at higher stoichiometries. These data established that once $polylysine_{19}$ DNA condensates are formed, they remained large throughout the titration range (Figure 2C).

Table 2. QELS Particle Size Distribution

peptide DNA condensate[a]	particle size population diameter[b] (nm)	σ[c] (nm)
$polylysine_{19}$	3102	297
$AlkCWK_3$		
$AlkCWK_8$	2412	354
$AlkCWK_{13}$	231	107
$AlkCWK_{18}$	78	30
$DiCWK_3$	724	154
$DiCWK_8$	53	24
$DiCWK_{13}$	56	29
$DiCWK_{18}$	64	27

[a] Peptide DNA condensates were prepared at a concentration of 20 μg/mL of DNA and at stoichiometry of 0.8 or 1.0 nmol ($DiCWK_3$) of peptide/μg of DNA in HBM. [b] Represents the mean diameter of particles. [c] Standard deviation of the population.

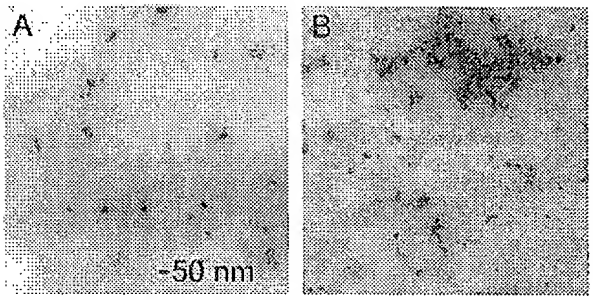

Figure 3. Electron microscopy of DNA condensates. The electron micrographs are shown for DNA condensates prepared at 0.5 nmol of peptide/μg of DNA for $AlkCWK_{18}$ (A) and at 0.8 nmol of peptide/μg of DNA for $polylysine_{19}$ (B). The calibration bar shown is 50 nm in length.

Particle Size and Distribution. DNA condensates were prepared with alkylated peptides, dimeric peptides, and $polylysine_{19}$ at a stoichiometry of 0.8 nmol of peptide/μg of DNA, and particle sizes were compared using quasi-elastic light scattering (QELS). A population of particles with average diameters of 0.7–3.1 μm was determined for $polylysine_{19}$, $AlkCWK_8$, and $DiCWK_3$ DNA condensates, whereas no particles were detected for $AlkCWK_3$ DNA condensates (Table 2), consistent with the results of sedimentation analysis.

Each alkylated or dimeric peptide possessing 13 lysine residues or more produced a population of particles with mean diameters of 53–231 nm (Table 2). It should be noted that particle populations were most often bimodal, possessing a major (>90%) smaller diameter population and a minor larger diameter population which contributed to the large standard deviation of the average particle size (Table 2).

Particle sizes determined by QELS were substantiated by analyzing DNA condensates using electron microscopy. Figure 3 compares the particle size and morphology for $AlkCWK_{18}$ and $polylysine_{19}$ DNA condensates. The images demonstrate that condensates produced with $AlkCWK_{18}$ are relatively uniform particles with diameters of approximately 50–100 nm, whereas $polylysine_{19}$-induced condensates were large flocculated particles, consistent with the result of particle size analysis by QELS (Figure 3).

***In Vitro* Gene Expression of Peptide DNA Condensates.** Luciferase reporter gene expression was analyzed following transfection of HepG2 or COS 7 cells with peptide DNA condensates prepared at stoichiometries ranging from 0.1 to 1.5 nmol of peptide/μg of DNA.

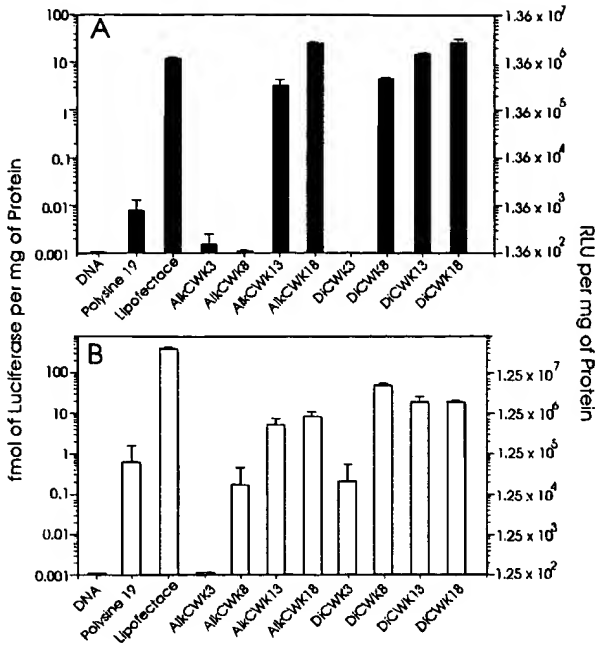

Figure 4. In vitro gene expression in HepG2 and COS 7 cells. Luciferase reporter gene expression is shown for DNA condensates prepared using alkylated peptides, dimeric peptides, polylysine$_{19}$, and Lipofectace. Chloroquine (80 μM) was included in the transfecting media for peptides and polylysine$_{19}$. (A) Gene expression determined in HepG2 cells; (B) gene expression in COS 7 cells. Each bar represents the mean and standard deviation of three determinations.

A 10-fold enhancement in the gene expression level was achieved when chloroquine was included in the transfecting media. For each peptide-condensing agent, the maximal reporter gene expression occurred at a peptide/DNA stoichiometry that corresponds to the fully condensed DNA as determined by the asymptote in the light scattering assay (Figure 1D,E,F) (*18*). At stoichiometries greater than that required to achieve condensation, the gene expression remained constant. Thereby, the relative gene expression levels were compared for each peptide DNA condensate at a fixed stoichiometry of 0.8 or 1.0 nmol (DiCWK$_3$) of peptide/μg of DNA, which was sufficient for each peptide to fully condense DNA.

Transfection of HepG2 with 10 μg of either uncomplexed plasmid DNA, AlkCWK$_3$ or AlkCWK$_8$, DiCWK$_3$, or polylysine$_{19}$ DNA condensates failed to produce significant reporter gene expression (Figure 4A). This result supported formulation experiments that predicted these peptides either fail to condense DNA (AlkCWK$_3$) or produce condensates that are large (0.7–3.1 μm). Alternatively, AlkCWK$_{13}$, AlkCWK$_{18}$, DiCWK$_8$, DiCWK$_{13}$, and DiCWK$_{18}$ DNA condensates each demonstrated significant gene transfer efficiency that was 2–3 orders of magnitude greater than that of polylysine$_{19}$. Lipofectace-mediated gene expression levels were also found to be identical to peptide-mediated expression levels in HepG2 cells (Figure 4A).

To verify that peptide-mediated gene delivery was not dependent on the existence of cell type specific receptors, the reporter gene expression in HepG2 cells was compared to that in COS 7 cells (Figure 4B). Significant differences were observed for the transfection of COS 7 versus HepG2 cells such that only uncomplexed DNA and AlkCWK$_3$ DNA condensates failed to produce measurable gene expression levels. AlkCWK$_8$, DiCWK$_3$, and polylysine$_{19}$ DNA condensates each mediated a significant gene expression in COS 7 cells despite their inactivity

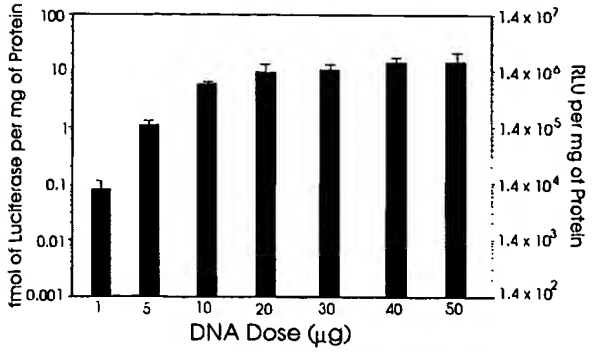

Figure 5. Dose response for peptide-mediated gene delivery. Luciferase gene expression levels in HepG2 are compared using an escalating dose of AlkCWK$_{18}$ DNA condensate prepared at a stoichiometry of 0.6 nmol/μg of DNA.

in transfecting HepG2 cells. However, the gene expression level mediated by these peptides was still 1–2 orders of magnitude below that afforded by AlkCWK$_{13}$, AlkCWK$_{18}$, DiCWK$_8$, DiCWK$_{13}$, and DiCWK$_{18}$ (Figure 4B). Also, Lipofectace-mediated gene expression in COS 7 cells was 1 order of magnitude greater than peptide-mediated gene delivery. These results suggest that the size restriction of peptide DNA condensates is less stringent in COS 7 cells compared to HepG2 cells.

To establish the effect of dose response using peptide DNA condensates, HepG2 cells were treated with escalating doses of AlkCWK$_{18}$ DNA condensates and Lipofectace DNA formulations. As demonstrated in Figure 5, a dose response curve for the AlkCWK$_{18}$ DNA condensate plateaus at 20 μg of DNA and remains constant at higher doses, whereas the toxicity of Lipofectace above 10 μg of DNA (data not shown) leads to reduced expression levels at higher doses.

DISCUSSION

The efficiency of carrier-mediated gene delivery depends on the reversible association of condensing molecules with plasmid DNA (*1–3*). The carriers that have been used most often are composed of polymers or lipids that bind to anionic sites on DNA. In the case of cationic peptides, this leads to condensation of plasmid DNA into small particles that gain entry into the target cell via nonspecific fluid phase pinocytosis (*1*). Attachment of ligands adds specificity to the delivery system and likewise alters the mode of DNA transfer across cell membranes such that DNA and ligand cotransfer via receptor-mediated endocytosis (*5–15*).

Previously we demonstrated that a low molecular weight glycopeptide mediated gene transfer to hepatocytes via the asialoglycoprotein receptor (*18*). Since this glycopeptide was prepared from low molecular weight polydisperse polylysine (dp 19), we sought to systematically optimize the peptide portion of the glycopeptide as a first step to improve its efficiency as a carrier for nonviral gene delivery.

The results establish that low molecular weight peptides possessing six or more lysine residues bind with sufficient affinity to condense DNA at stoichiometries above the charge neutral point (Figure 1). However, condensation of DNA is not sufficient to ensure significant transfection levels since AlkCWK$_8$, DiCWK$_3$, and polylysine$_{19}$ each produced DNA condensates but failed to mediate significant gene transfer in HepG2 cells (Figure 4A). The success or failure of individual peptide DNA condensates to mediate gene expression appears to be related to particle size such that larger condensates

are less efficiently pinocytosed (*2*). For HepG2 cells an apparent size restriction exists that excludes large DNA condensates. This is demonstrated by comparison of the transfection efficiency for $AlkCWK_8$ DNA condensates of 2.4 μm size relative to $AlkCWK_{13}$ DNA condensates, which possess an average diameter of 231 nm (Figure 4A). The addition of five lysine residues decreases particle size 10-fold, which leads to a 1000-fold amplification in gene transfer efficiency. Further reductions in the DNA particle diameters in the range of 231−53 nm only led to an additional 10-fold increase in transfection levels (Figure 4A). The size requirements described above for transfecting HepG2 cells are less stringent for COS 7 cells. Large (0.7−3.1 μm) peptide DNA condensates are moderately efficient at mediating transfection in COS 7 cells but are still 10−100-fold less efficient than smaller DNA condensates (Figure 4B).

The equivalent transfer efficiency of peptide DNA condensates into either HepG2 or COS 7 cells suggests a nonspecific mechanism related to the cationic nature of the condensates (*1*, *2*). We have also transfected 293T cells with peptide DNA condensates (data not shown), which resulted in a similar high level of gene expression. Comparable gene expression levels were obtained using peptide or Lipofectace to mediate DNA transfer in HepG2 cells, whereas Lipofectace-mediated gene delivery was found to be more efficient that peptide-mediated gene delivery in COS 7 cells (Figure 4). These results reflect cell type specific differences that must be considered in the development of gene delivery systems (*1*, *2*).

The discovery of a class of low molecular weight peptides that efficiently condense DNA into small particles is a major finding of this study. Apparently, some structural feature of $AlkCWK_n$ peptides allows more efficient condensation of DNA relative to polylysine. This is demonstrated most clearly by $AlkCWK_{18}$, which condenses DNA into particles that are 40-fold smaller (78 nm) than those produced by $polylysine_{19}$ (3.1 μm). Given that the lysine chain lengths of these two peptides are nearly equivalent, the N-terminal cysteine or tryptophan is presumably responsible for the enhanced condensing activity. To investigate the structural requirements of an efficient DNA condensing peptide, we synthesized an isomer of $AlkCWK_{18}$ also possessing 18 lysine residues but in which cysteine is relocated to the C terminus and tryptophan is the N terminus ($WK_{18}C$). The alkylated and dimeric form of this peptide each mediated gene transfer as efficiently as $AlkCWK_{18}$ (data not shown), suggesting that the location of cysteine may not be key to the reported activity. On the basis of this and other observations discussed below, we hypothesize that tryptophan may be primarily responsible for the enhanced condensation activity of $AlkCWK_{18}$ compared to $polylysine_{19}$.

A tryptophan residue may increase the binding affinity between cationic peptides and DNA. Evidence supporting this hypothesis comes from the stoichiometry of $AlkCWK_{18}$ (0.2 nmol of peptide/μg of DNA) needed to exclude intercalator binding to DNA versus that for $polylysine_{19}$ (0.6 nmol of peptide/μg of DNA) (Figure 1A,C). Lohman and co-workers also identified a function for tryptophan in altering cationic peptide binding to DNA and RNA (*22*, *23*). Curiously, they determined an enhancement in the entropy of peptide binding to DNA when substituting tryptophan for lysine (*22*, *23*). However, this was offset by a decrease in the enthalpy of binding, leading to a net zero change in the association constant (*22*, *23*). These studies also established that the location of tryptophan is not important and that multiple tryptophan residues do not influence the magnitude of the association constant despite changes in the enthalpic and entropic contributions (*22*, *23*). Recently, a low molecular weight cationic peptide possessing alanine, tyrosine, 10 lysines, and tryptophan has been co-complexed with a fusogenic peptide and DNA to achieve a 5 order of magnitude amplification in gene expression in HepG2 cells relative to uncomplexed DNA (*24*). The efficient DNA condensing activity of this low molecular weight peptide may also be linked the tryptophan residue, which flanks the polylysine sequence.

The precise mechanism of how tryptophan functions to increase binding affinity and decrease particle size is uncertain; however, it may relate to its ability to intercalate into DNA leading to an observed fluorescence quench (*21*−*23*). Tryptophan's hydrophobic interaction with DNA may organize the peptide binding on DNA, facilitating the formation of intermolecular ion pairs between multiple lysine residues and the DNA phosphate backbone.

Preparation of small DNA condensates has also recently been reported using polylysine dp 30 at a stoichiometric excess of 1.2 nmol/μg of DNA (*20*). The present study establishes that simple amino acid substitutions allow polylysine peptides as small as dp 13 to acquire the necessary affinity to condense DNA into small particles at low stoichiometric excess.

The development of homogeneous peptides that actively condense DNA into small particles is an important advance toward the development of low molecular weight carriers for targeted gene delivery. Attachment of a receptor ligand such as a carbohydrate or peptide to a single cysteine residue should endow specificity to the gene delivery system and allow further systematic optimization of low molecular weight carriers for receptor-mediated gene delivery.

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1: Mahat RI, Monera OD, Smith LC, Rolland A.Peptide-based gene delivery.
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Peptide-based gene delivery

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To achieve effective plasmid-based gene therapy, the control of cellular access and uptake, intracellular trafficking and nuclear retention of plasmids must be achieved. Inefficient endosomal release, cytoplasmic transport and nuclear entry of plasmids are amongst some of the key limiting factors in the use of plasmids for effective gene therapy. A number of non-viral gene delivery systems have been designed to overcome these limiting factors. The most common approach to protect and control plasmid distribution is to complex plasmids with cationic lipids or polymers through electrostatic interactions. Endosomal release of plasmids can be achieved, for instance, by using pH-sensitive lipids, inactivated viral particles, endosomolytic peptides and polymers. Among the least explored gene delivery systems are those that consist mainly of synthetic, short peptides. Peptides can be incorporated into multi-component gene delivery complexes for specific purposes, such as for DNA condensation, cell-specific targeting, endosomolysis or nuclear transport. The aims of this review are to: (i) explore the conceptual and experimental aspects of peptide-DNA interactions; (ii) critically assess the possible use of peptides for efficient gene transfer; and (iii) present an overview on the use of peptides to enhance the effectiveness of other gene delivery systems. On balance, peptide-based gene delivery systems appear to have a significant potential as commercially viable gene delivery products.

Introduction

Non-viral gene delivery systems have recently generated considerable research interest because of many inherent advantages over the viral gene delivery systems in terms of safety, immunogenicity, and ease of manufacture. In the design of gene carrier systems, scientists have used synthetic reagents that emulate components of viruses as models for efficient plasmid delivery. The four intrinsic viral attributes that have attracted the most attention are DNA condensation, interaction with target cell surface receptor, membrane fusion and nuclear localization. Viral particles containing condensed viral DNA specifically bind to receptors on the surface of selected cells and get internalized. The membrane fusion proteins enable escape of the viral genome from the endosomal compartment into the cytoplasm or direct transport through the plasma membrane. Finally, transport of viral DNA across the nuclear membrane is facilitated by proteins containing nuclear localization sequences (NLS). Thus, an ideal non-viral gene delivery system may also contain DNA condensing, receptor-binding, endosome-disrupting, and nuclear-targeting elements [1,2,3••,4•,5]. The most common delivery systems to date utilize: (i) polycations, such as polylysines, polyarginines, spermine, spermidine, dendrimers, chitosans and polyethyleneimine to condense the extended structure of plasmid into a small, tightly packed complex by ionic interactions [6-9,10•,11,12]; and (ii) liposomes to either encapsulate [13] or form complexes with plasmid [14]. To confer cell specificity, targeting ligands are often covalently attached to these cationic polymers, or anchored to the liposomes [15]. Plasmid release into the cytoplasm may be facilitated by the ability of the liposome to fuse with the endosomal membrane. NLS peptides can also be incorporated into plasmid carrier complex to increase nuclear uptake of the plasmids [16,17]. Some of the major disadvantages of both cationic lipid- and polymer-based systems can be their relative toxicity and low gene transfer efficiency. These two gene delivery systems have been extensively reviewed elsewhere and will not be dealt with in this review.

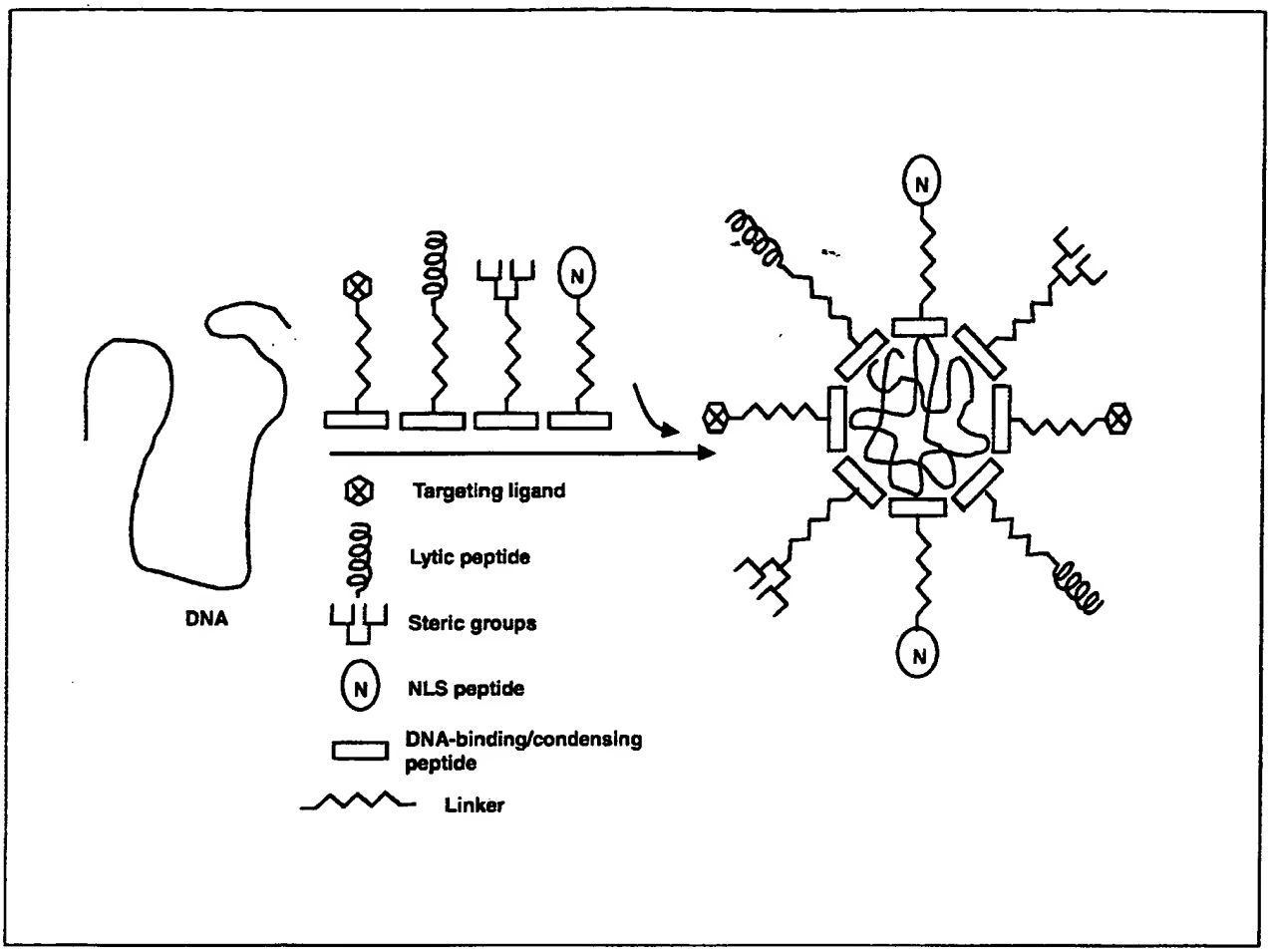
Peptide-based gene delivery systems that incorporate all four viral attributes seem to be the least developed, and will be discussed exclusively in this review. It is known that the active sites of enzymes, receptor ligands and antibodies usually involve about 5 to 20 amino acids [18••]. Thus, it should be possible to design small synthetic peptides to mimic the active sites of viral proteins and formulate synthetic peptide/plasmid complexes that are as efficient as viruses, but do not have their limitations. There has been some skepticism about the potential success of a peptide-based gene delivery system, mostly based on the idea that electrostatic interactions between the plasmid and the peptide are not stable enough under physiological ionic conditions. However, this review will show that peptides with well-defined structural and chemical properties have been designed to address the different requirements of an 'idealized' non-viral gene delivery system.

The aims of this review are 3-fold. The first is to explore the conceptual and experimental aspects of DNA and peptide interactions, and present insights as to how these interactions can be utilized to design peptide-based gene delivery systems. The second is to identify the extracellular and intracellular limitations that need to be overcome by a non-viral gene delivery system, and how peptides can be designed to address these limitations. The final objective is to present an overview of gene delivery systems based on other carrier systems in combination with peptides to enhance their effectiveness.

Design elements of peptide-based gene delivery systems

The design elements of peptide-based gene delivery systems are illustrated in Figure 1. To construct an 'ideal' gene delivery system, different peptides have to be designed to address the different barriers that the plasmid has to overcome before becoming transcriptionally active in the nucleus. The major components of peptide-based delivery systems are: (i) a DNA binding peptide to condense the plasmid; (ii) a targeting peptide to confer cell and/or tissue specificity; (iii) a lytic peptide to induce endosomal release of the plasmid; and (iv) an NLS peptide to enhance nuclear entry of the plasmid.

Figure 1. The design elements and assembly of a peptide-based gene delivery system.



The major components of a peptide-based delivery systems are: (i) DNA binding peptides to condense DNA as well serve as templates for conjugating other ligands; (ii) a targeting peptide ligand for cell-specific targeting; (iii) a lytic peptide to induce endosomal release of plasmid; (iv) an NLS peptide to enhance nuclear entry of plasmid; and (iv) an optional steric group to confer stability in biofluids.

DNA condensation

Recent progress in the understanding of DNA condensation [19•,20••] includes the observation of DNA collapse, greater insights into the intramolecular forces driving condensation, the recognition of helical structure perturbation in condensed DNA and the increasing recognition of the biological consequences of condensation. The extent of DNA condensation is highly important for gene delivery and expression. The same physical forces that are required for DNA condensation must be reversed for the release of the plasmid within the large cell to make it available for cellular transcriptional mechanisms that lead to gene expression.

Reversible DNA condensation into small particles is one of the major obstacles for efficient gene transfer for several disease targets. For example, the DNA of bacteriophage T4 in dilute solution has a radius of gyration of about 1000 nm, but it is reduced to only a 50 nm particle inside the T4 phage head [21]. Clearly, this dramatic reduction in particle size is a pre-requisite for efficient DNA packaging and delivery. Although *in vitro* attempts to condense DNA by addition of multivalent cations have produced a comparable size reduction [22], a structurally and morphologically distinct condensed state has been difficult to distinguish from mere aggregation or precipitation.

In this review, the term DNA condensation will be used specifically to refer to a process by which the volume occupied by the DNA molecule is decreased by the formation of a compact state of finite size and morphology in which the volume fractions of solvent and DNA are comparable [19•,20••]. The most common condensed forms are toroids, spherical globules, rods and liquid crystal. In the best-characterized toroidal structures, the DNA duplex circumferentially wraps around itself to form an inner radius of around 7.4 nm and an outer radius of 45 nm [22]. Each toroidal structure appears to settle into a stable complex of six units. Apparently, 400 base pairs (bp) is the minimum length of DNA necessary to form the nucleation site for toroid formation [23]. However, above the minimum length, the toroid size is invariant with the DNA size, suggesting that the toroidal particle may contain one large DNA or several small DNA molecules [21,24]. Not surprisingly, DNA topology influences both the colloidal

behavior and condensation of DNA. For instance, supercoiled plasmids yield smaller toroids than linear DNA [20••]. In addition, when closed circular plasmid was condensed with hexamidine cobalt(III), it yielded multimolecular toroids that were 25 to 30% smaller in diameter than that made of linearized plasmid [25].

While DNA condensation from aqueous solutions generally produces a toroidal structure, condensation in alcohol generally produces more rod-like structures [26] especially when provoked with hexamidine cobalt [27] or permethylated spermidine [28]. In addition to solvent polarity, the mixing procedure can also produce a different morphology. Globular complexes are preferentially formed when plasmid is mixed vigorously with polylysine or histone [20••].

DNA condensation is a result of a complex interplay of different interactions, many of which are not well understood. However, important insights are emerging. Multivalent cations are presumed to condense DNA by neutralizing the negative charges of the phosphate groups and, therefore, decreasing the coloumbic repulsion between the DNA phosphates and increasing hydrophobic interactions of the complexed sites [20••,21]. At least 90% of the DNA charge must be neutralized for condensation to occur [20••,24]. The electrostatic interaction between the negatively charged DNA backbone and the multivalent cations causes displacement of most water molecules and reorientation of the remaining water dipoles near DNA surfaces. This in turn may allow local alignment of helical segments in which DNA helices may be separated by just one or two layers of water molecules. In most cases, the volume fractions of water and DNA have been found to be comparable [20••]. However, the observation that the addition of solvents, such as ethanol [24,26,27], or another polymer, such as polyethylene glycol [29], also results in DNA condensation indicating the occurrence of other poorly understood mechanisms. Even anionic polymers, such as polyaspartate or polyglutamate have been reported to provoke DNA condensation [30]. In these cases, DNA condensation is believed to be due to decreased interaction between the DNA and the solvent, ie, water.

The precise structural elements that are necessary for the multivalent cation to be efficient for DNA condensation are unknown. However, there is considerable evidence that the cationic charges have to be clustered because most monovalent and divalent cations do not condense DNA, except under special circumstances where they may act synergistically with other condensing agents [20••]. Although small multivalent cations bind DNA, they are highly mobile and can easily be displaced by compounds with a higher charge density. DNA condensation normally requires clusters of three or more cationic charges [20••,24,25]. This is consistent with the observation that cationic detergents condense DNA only above their critical micellar concentration (CMC), suggesting that the monovalent cationic species have to be first clustered into a micellar or membrane-like structure before they can condense DNA [31]. These observations clearly support the contention that, in addition to the reduction of the coloumbic repulsion, attractive free energy may also come from other forces, such as the bridging of the charges by the clustered condensing moieties, the formation of interhelical interaction between bases that are normally disrupted by the solvent, and increased hydrophobic interactions as an indirect effect of charge neutralization. It has been estimated that the attractive force that holds the plasmid in the condensed state is about 0.015 kcal/bp [32].

Apart from the clustering of polycations, other structural elements such as hydrophobicity of the polycation and spatial arrangement of the charges are important. For example, when a series of diaminoalkanes ($NH_3^+(CH_2)_nNH_3^+$, where n = 1 to 6) were tested for DNA condensation, analogs with n = 3 and n = 5 condensed DNA, while those with n = 2, n = 4 and n = 6 did not [33]. In addition, the nature of the uncharged residues is also important because cationic peptides containing three or six repeats of the tetrapeptide units Leu-Ala-Arg-Leu were observed to bind and condense DNA, but the binding was significantly reduced when some of the Leu residues were substituted for Ser [34]. Also, polycationic peptides showed improved DNA binding and condensation when tryptophan was added to the sequence, which may be due to the increase in hydrophobicity of the peptide or possibly a more specific role of Trp in intercalating into DNA to provide a more ordered condensation process [35•,37,145••]. The nature of the basic amino acid as well as the carboxy-terminal modification of the condensing peptide can have significant influence on its gene transfer efficiency. For example, in a series of acylated short polylysine and poly(α,γ-diaminobutyric acid), containing either a methyl ester or hydrazide carboxy termini, the hydrazide forms consistently gave up to 20-fold higher transfection activity [37]. This result has been attributed to increased DNA binding affinity through more favorable hydrogen bonding between the DNA and the hydrazide carboxy-terminus. Furthermore, the length of fatty acid chains also plays an important function, since shortening of the acyl chain from 16 to 6 carbon atoms completely abolished transfection. Finally, the inclusion of a disulfide bond between short Lys clusters has also been reported to enhance DNA-binding and transfection [36].

In attempts to capitalize on the requirement for clustered positive charges to condense DNA, various polyamines of different lengths and structures have been used, including polylysines, polyarginines, spermidine, spermine, dendrimers, protamines, polyethyleneimine and poly(α,γ-diaminobutyric acid) [6-9,10•,11,12,38•]. However, these long clusters of positive charges may not be necessary and often cause irreversible DNA precipitation. Kabanov and Kabanov [38•] suggested that co-operative binding to DNA limits polylysine binding in complex formation. These authors estimated that the maximum length of polyion sites for co-operative binding is approximately 10 polyion units. Gotchalk *et al* [35•] investigated several synthetic polylysine analogs within this 10 polyion range and concluded that Tyr-Lys-Ala-Lys_6-Trp-Lys was an optimal DNA condensing sequence. In their subsequent study [39•] where the number of core lysine residues in the peptide Tyr-Lys-Ala-$(Lys)_n$-Trp-Lys were varied from n = 4 to n = 40, they observed a clear increase in transfection efficiency with the increase in number of core Lys residues, which leveled off at around n = 7 or 8. This increase in transfection was attributed to the

increase in binding of the peptide to DNA as the length of the polycationic cluster increased. However, longer analogs (n = 12 and n = 40) were less efficient in transfection, probably due to cytotoxicity generated by the large number of lysine residues. From these results, a mathematical expression was generated which defines the number of core lysine residues for optimizing particle sizes and transfection efficiency. Further studies showed that substitution of the lysine residues with other cationic residues reduced activity [18]. However, neither length nor specificity of the basic amino acid is an absolute requirement because condensation of DNA with as few as three Arg residues has been reportedly achieved [34].

All of the above observations indicate that DNA condensation is a complex process, considerably more than simple charge neutralization of the phosphate groups. However, a variety of DNA condensing peptides have been designed (Table 1) and shown to improve transfection efficiency. These peptides are amenable to further functional refinement to achieve fine control of the DNA condensation process that results in well-defined, self-assembling gene delivery systems. Other possible structural fine-tuning of the cationic clusters include linear polylysine versus branched or multiple antigenic peptide (MAP)-type polylysines, α-linked polylysines versus ε-linked polylysines, linear polylysine versus poly(α,γ-diaminobutyric acid), polylysine versus poly(Lys-Gly) and primary amines versus guanidine amines, as examples of other structural variations.

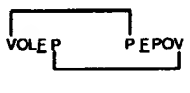
Targeting ligand

Gene delivery can broadly be categorized as passive and active targeting. Passive targeting refers to the biodistribution of particulate formulations, as determined by their physicochemical properties, their interaction with biofluids and the anatomical features of the body. By contrast, active targeting refers to alteration in the natural disposition pattern of plasmids by means of target-specific ligands, which can interact specifically to the surface of selected cells [2•].

Site-specific delivery of plasmids requires the identification of cell surface receptors and the design of appropriate ligands for receptor-mediated endocytosis. High selectivity and affinity are essential features for efficient targeting of the DNA complex. Previously used receptor ligands have been either native molecules or their modified forms. Common examples of these include ligands for the asialoglycoprotein receptor, transferrin, polymeric immunoglobulin, insulin, epidermal growth factor (EGF), lectins, folate, malaria circumsporozoite protein, α_2-macroglobulin, CD3-T-cell, sugars, integrins, thrombomodulin, surfactant protein A and B, mucin and the c-kit receptor [40-57]. However, coupling of multiple copies of these large native ligands, which are mostly proteins, has some inherent disadvantages. For example, in addition to being poorly defined molecularly, they can drastically alter the size and structure of the plasmid complex, mask other ligands in the complex, alter solubility and induce other changes that can potentially decrease the efficiency of delivery. Ideally, the chosen ligand should be incorporated into the formulation with minimal loss of affinity and specificity and should be readily released from the complex after receptor-mediated internalization of the complex into the cells, or shortly thereafter.

Because of the potential problems mentioned above, there is now a growing interest in the use of smaller ligands that are derived only from the active site or specific epitopes of the native ligand. The major advantages of small peptides or epitopes include ease of design, synthesis and conjugation, their resistance to proteolysis and their weak immunogenicity. The most well-characterized example is the Arg-Gly-Asp (RGD)-containing peptides. This sequence is found in many extracellular matrix proteins, such as vitronectin, laminin, fibrinogen and fibronectin. RGD-containing peptides have

Table 1. Typical endosomolytic and condensing peptides.

Peptide Name	Structures	References
Condensing peptides		
K_8	YKAK_8WK	Gottschalk *et al* [35]
ALK-CWK_8	S-(carboxymethyl)-CWK1$_8$	Wadhwa *et al* [37]
GM212.8	Nα,Nε-dipalmitoyl-KK_8WK	GENEMEDICINE unpublished
N-acyl-(diaminoobutyric acid)2-hydrazide	N-palmitoyl-(Dab)$_2$-CONHNH$_2$	Legendre *et al* [38]
Endosomolytic and condensing peptides		
KALA	WEAKLAKALAKALAKHLAKALAKALKACEA	Wayman *et al* [138]
Gramicidin-S		Legendre and Szoka [145]
Dioleoylmelittin	S-dioleoyl-CIGAVLKVLTTGLPALISWIKRKRQQ	Legendre *et al* [145]
Endosomolytic peptides		
GALA	WEAALAEALAEALAEHLAEALAEALEALAA	Haensler and Szoka [89]
INF-1	GLFEAIAGFIENGWEGMIDGGGC	Wagner *et al* [78]
JTS-1	GLFEALLELLESLWELLEA	Gottschalk *et al* [35]
GM227.3	GLFEALEELWEAK(ε-G-dipalmitoyl)	McLaughlin *et al* [11]

Standard one-letter designation is used in all peptide sequences and the underlined letters in the sequence represent D-amino acids.

been shown to inhibit integrin binding to its receptor. Detailed structure-function studies [58,59] of cyclic pentapeptides containing the sequences cyclic Arg-Gly-Asp-D-Phe-Val and cyclic Arg-Gly-Asp-Phe-D-Val and showed that a hydrophobic residue after the Asp, as well as the proton of the amide bond between Asp and the subsequent amino acid, are important for strong inhibition of the vitronectin-$\alpha_v\beta_3$ interaction. Phage displaying an RGD sequence was also shown to bind to the integrins present on tumor cells when injected intravenously into tumor-bearing mice [60•], demonstrating the target-specificity of RGD sequence when linked to macromolecular assemblies. The potential of utilizing an RGD containing peptide in targeting plasmids to different cell lines is now beginning to be exploited [61].

Another targeting approach is to use a non-peptide targeting ligand, such as glycoside, as a covalently attached moiety of a DNA condensing peptide, or other components, of the plasmid complex. For example, when various triantennary oligosaccharides were coupled to short polylysines (dp = 19) and used in plasmid formulations, the plasmid/glycopeptide complexes were endocytosed into HepG2 cells [62,63], presumably via the asialoglycoprotein receptor. Similarly, formulations containing pCAT plasmid, a lipolytic peptide and a lipophilic DNA condensing peptide with galactose coupled to each of the three branch points were preferentially targeted to rat liver hepatocytes [200]. Furthermore, plasmid formulations containing tetragalactosyl neoglycopeptide were shown to lead to reporter gene expression in hepatocytes [64]. Similarly, galactosylated and mannosylated polylysines (~2000 Da) have been reported to increase gene delivery and expression in rat hepatocytes [65] and in murine macrophages [66], respectively. These results demonstrate that small glycosides conjugated to peptides can facilitate DNA targeting and delivery similar to the native asialoglycoproteins.

Endosomolysis

Once the plasmid/carrier complexes have been taken up by the cells via adsorptive or receptor-mediated endocytosis, the plasmid must escape the lysosomal degradation and be released into the cytoplasm. Endosomal membranes contain an ATP-dependent proton pump, which decreases the internal pH of the endosome. Many viruses release their viral genome through the action of a viral protein, which disrupts the endosome membranes by lysis and/or fusion. Therefore, the general strategy is to exploit endosomal acidification to achieve endosomolysis before the contents of the endosome are delivered to the lysosome. For example, pH-sensitive liposomes, inactivated viral particles and fusogenic or endosomolytic peptides have been used to facilitate the release of plasmids into the cytoplasm prior to lysosomal degradation [67,68••].

The most characterized synthetic peptides with pH-dependent fusogenic activity are derived from the first 23 amino acids of the N-terminal peptide of the HA2 subunit of influenza hemaglutinin and are referred to as INF peptides (Table 1). At pH 7 these peptides assume a random coil structure which has essentially no lytic activity. As the pH decreases, the carboxyl groups of aspartate and glutamate side chains become protonated, and the loss of repulsive anions allows the transition from a random coil into an amphipathic α-helical conformation that can interact with phospholipid membranes to induce fusion and/or lysis. At pH 5 the peptides become endosomolytic. With some peptides, the increase in transfection *in vitro* is as much as 100-fold [69,70].

Although there seems to be no close relationship between sequences of many virus-derived and comparable synthetic membrane active peptides, they share a number of structural and functional similarities. These sequences are generally 15 to 30 residues long and contain alternating clusters of hydrophobic residues and a hydrophilic amino acid such that, when arranged into an α-helical structure, an amphipathic helix is usually revealed. However, it should be noted that not all amphipathic peptides are membrane active. These membrane active sequences are usually found at the N-terminus of the viral protein and contain several acidic residues (Glu or Asp) on the hydrophilic face.

Membrane destabilization by endosomolytic peptides is thought to occur by two mechanisms: membrane rupture and pore formation. Membrane rupture results from the alteration of the membrane properties by the peptide. The membrane destabilizing α-helical peptides generally have a narrow hydrophilic face, and a wide hydrophobic face that facilitates burial of the peptide into the hydrophobic phase of the membrane bilayer. This, in turn, increases the negative curvature strain of the lipid bilayer, causes more general disruption of the lipid packaging, and facilitates transition from the bilayer phase to the hexagonal phase. The ultimate consequence of this rearrangement is the formation of leaky patches in the membrane [71••]. Examples of this type of peptide include magainin, mastoparan and the N-terminus of SIV [74]. Conversely, amphipathic α-helical peptides that have wider hydrophilic face are generally membrane stabilizing and therefore, have poor lytic activities.

On the other hand, pore formation results when the peptides self-assemble across the membrane bilayer to form transmembrane pore without affecting general membrane properties. As a consequence of pore formation, transmembrane potential and osmotic swelling can lead to lysis of the cells. The postulated sequence of events starts from the binding of the α-helical peptide monomer to the membrane surface, initially through electrostatic interactions, followed by oblique insertion of the peptide into the hydrophobic phase of the membrane bilayer, reorientation of the peptide perpendicular to the membrane surface, migration of the peptide along the membrane, and ultimately aggregation of several peptide helices into a barrel-like structure with a central aqueous pore [72]. Alternately, the peptide monomers may already associate with each other, at least to some extent, before total insertion into the membrane. It is not clear what controls the degree of aggregation and the final size of the pores but results in the leakage of vesicle contents in a manner that depends on the size of the solute. By contrast, for the membrane-disrupting peptides, there should not be size-dependent release of solute [73]. The peptides also induce rapid exchange of phospholipids in the membrane bilayers, the rate of which is dependent on amino acid composition and residue location in the pore. Examples of peptides that are believed to promote pore formation are alamethacin, pardaxin or Bti toxin helix-2, melittin and probably the INF type of lytic peptides [74,75].

Wagner and colleagues [76,77] utilized the pH-dependent lytic activity of the INF peptides to achieve endosome lysis and enhance gene delivery. Their results showed that when these INF peptides, containing different analogs of the first 19 to 23 amino acid residues of influenza H2A amino terminus, were mixed with polylysines and complexed with plasmid, different activities were obtained, ranging from 10- to 10,000-fold enhancement of luciferase transfection *in vitro*. More interestingly, dimers formed by disulfide bond formation between two amino terminal cysteines were 1000-fold more active than the corresponding monomeric peptides. When the INF peptides were incorporated non-covalently into polyethyleneimine/DNA [78] and cationic lipid/DNA [79•] complexes, up to 10- and 10,000-fold enhancement in transfection was observed, respectively.

A fully synthetic pore-forming agent developed by Gottschalk *et al* [35], called JTS-1, contains the amino acid sequence Gly-Leu-Phe-Glu-Ala-Leu-Leu-Glu-Leu-Leu-Glu-Ser-Leu-Trp-Glu-Leu-Leu-Leu-Glu-Ala. The hydrophobic face contains only strongly apolar amino acids, while negatively charged glutamic acid residues dominate the hydrophilic face at physiological pH. Based on molecular modeling studies, the hydrophobic face of JTS-1 causes self-association and forms pores in one side of the endosomal membrane, thereby destabilizing the membrane and leading to its rupture. When negatively charged JTS-1 was rapidly mixed with cationic DNA complexes formed with the condensing peptide Tyr-Lys-Ala-Lys_8-Trp-Lys (K_8), a ternary complex spontaneously formed through electrostatic interactions. At a given charge ratio of condensing peptide to plasmid, the transfection efficiency has been shown to be proportional to the concentration of the endosomolytic peptide added to the complex. *In vitro* transfection efficiency was up to 10,000-fold higher than that of DNA/Tyr-Lys-Ala-Lys_8-Trp-Lys complex alone. In order to supplement the salt-sensitive electrostatic interactions between JTS-1 and K_8, dipalmitoylated analogs of each peptide have been synthesized to provide stabilizing hydrophobic interactions. Plasmid formulations containing these diacylated peptides yielded smaller particle sizes that were active and stable to salt and serum challenge [Valentis Inc, unpublished data].

Additional amphipathic α-helical peptides with pH-dependent lytic activities have also been designed *de novo*. It is known that the formation and stability of amphipathic α-helices can be controlled by the inclusion of amino acid residues with appropriate α-helical propensities [80,81]. For example, α-helices can be stabilized by inclusion of more Leu and Ala residues or, conversely, destabilized by inclusion of helix breaking Gly and Pro residues. Furthermore, pH dependence of the α-helical structure can be modulated by correct positioning of the charged residues in the helix [82]. Consistent with these principles, Szoka and colleagues [83-86] pioneered the design and synthesis of the 30 amino acid residue amphipathic peptide, GALA, based on the repeat unit Glu-Ala-Leu-Ala (Trp-Glu-Ala-Ala-Leu-Ala-Glu-Ala-Leu-Ala-Glu-Ala-Leu-Ala-Glu-His-Leu-Ala-Glu-Ala-Leu-Ala-Glu-Ala-Leu-Glu-Ala-Leu-Ala-Ala). This peptide also exhibits the pH-dependent transition from a random coil structure at pH 7 to an amphipathic α-helix at pH 5, which strongly interacts with the membrane to induce fusion and leakage of the contents. When GALA was covalently attached to polyamidoamine cascade polymers [87] or non-covalently associated with positively charged polylysine/DNA complexes [88], transfection efficiencies were increased by 2- to 3-fold *in vitro*.

An interesting observation has recently been published [89•] which, instead of exploiting the protonation of the acidic amino acid residues, is based on the protonation of the imidazole ring of histidine. Under specific experimental conditions, the peptide called H5WYG with the amino acid sequence of Gly-Leu-Phe-His-Ala-Ile-Ala-His-Phe-Ile-His-Gly-Gly-Trp-His-Gly-Leu-Ile-His-Gly-Trp-Tyr-Gly was not active at pH 7, induced 50% cell leakage at pH 6.8, and optimally permeabilized cells at pH 6.4. The unique feature of this peptide is due to the very narrow pH range where structural transition occurs, and the pH of transition is closer to physiological conditions. Interestingly, the proposed mechanism of membrane perturbation by H5WYG is the reverse of that postulated for the INF peptides. In H5WYG, the α-helical structure is inactive at pH 7, while the random structure is active at a slightly acidic pH. In the INF peptides, the helical structure at acidic pH is active while the random structure at pH 7 is inactive. But their common property is that both become active in membrane perturbation upon acidification of the endosome. The H5WYG peptide is presumed to insert into the endosome membrane at pH 7, and upon acidification the polycationic species generated by the protonation of His cause membrane perturbation which leads to eventual leakage [89•]. However, it is not clear if the DNA can be readily released from the protonated H5WYG because of the enhanced electrostatic interactions.

Several plasmid formulations containing condensing peptides and lytic peptides have been shown to transfect cells *in vitro*. In general, gene transfer efficiency is much better in rapidly dividing cells than in post-mitotic cells. For example, Wilke *et al* [90] developed a peptide-based system which consists of a 12 amino acid DNA binding peptide (Ser-Pro-Lys-Arg-Ser-Pro-Lys-Arg-Ser-Pro-Lys-Arg) with a palmitoyl group added at the amino terminus, and a fusogenic peptide (Gly-Leu-Phe-Glu-Ala-Ile-Glu-Phe-Ile-Glu-Gly-Gly-Trp-Glu-Gly-Leu-Ile-Gly-Cys) derived from the hemagglutinin protein. The peptide/plasmid complexes prepared using these peptides and a luciferase plasmid produced higher levels of reporter gene expression in various rapidly growing cell lines, compared to cationic liposome-based formulations. Another important observation was that transfection efficiency was dependent on mitotic activity, as cells that were prevented from going into mitosis after transfection expressed the marker gene much less efficiently than proliferating cells. In search of an explanation for this phenomenon, the authors studied the transport of plasmid across the nuclear membrane. Plasmid injected into the cytoplasm of quiescent human fibroblasts was not expressed, in contrast to plasmid injected into the nucleus. This was also true for the cationic lipid-based systems, as plasmid injected into the cytoplasm of *Xenopus* oocytes was not expressed, in contrast to plasmid injected into the nucleus [91], indicating that the plasmid must dissociate from the cationic lipids before entering into the nucleus. To this end, anionic lipids normally found on the cytoplasmic-facing monolayer of the cell membrane can potentially displace plasmids from lipid/plasmid complexes

[92•]. Taken together, these findings suggest that the nuclear translocation of plasmid in non-mitotic cells is probably one of the highest barriers to transfection that needs to be overcome [93].

Nuclear localization

Trancription can only take place when the uncoated plasmids are present in the nucleus [94]. A distinctive feature of eukaryotic cells is a nucleus that is enclosed by the nuclear envelope. Macromolecular exchange between the cytoplasm and the nucleus is generally regulated by a large number of nuclear pore complexes (NPCs) in the nuclear membrane. A number of excellent reviews on the morphology of the NPC and the molecular mechanisms of nuclear transport are available [95,96•,97•]. The NPC is composed of more than 100 proteins and polypeptides with an estimated aggregate mass of 125 megaDaltons. The NPC has an inner pore that allows free diffusion of molecules < 9 nm in diameter [95]. For example, cytochrome C (13 kD) diffuses freely through the pore, while diffusion of ovalbumin (43 kD) is delayed and that of bovine serum albumin (64 kD) is virtually prevented. Passive entry of larger proteins does not occur and their selective entry into the nucleus through an active transport process that is facilitated by nuclear localization signals (NLS) [95].

Transport across the nuclear pore complex is believed to involve the following discrete steps: (i) binding of the NLS-bearing macromolecule with cytoplasmic nuclear protein receptors; (ii) docking of the NLS-receptor complex into the cytoplasmic periphery of the pore; (iii) ATP-dependent fibril-mediated translocation of the molecules over the central transporter channels across the lumen of the pore complex; and (iv) release to the nucleus [95,96•]. Therefore, understanding the nuclear transport process is essential to devise strategies to achieve efficient plasmid delivery to the nucleus.

The NLS are short amino acid sequences that are recognized by cytoplasmic transport receptors called karyopherins or importins. The putative NLS sequences have been deduced from the amino acid sequence of proteins by either or both 'subtractive' and 'additive approaches' [99]. The first approach involves either deletion or mutation of a putative NLS sequence to demonstrate that no nuclear transport of a particular protein occurs. The result of the first approach is then confirmed by the second approach, wherein the putative NLS sequence is added to a non-nuclear protein to demonstrate nuclear localization. The NLS sequences identified so far, generally contain one (monopartite) or two (bipartite) clusters of four or more basic amino acids (Lys and Arg), although there are many exceptions and variations. However, the following generalizations can be drawn from their sequences: (i) α-helix-destabilizing Pro or Gly residues are often found upstream or downstream of the cluster of basic residues; (ii) hydrophobic residues (Trp or Tyr) are rarely found either in the cationic cluster or in the spacer of the bipartite NLS sequences; (iii) clusters of acidic residues upstream, downstream or sometimes in the spacer sequence are thought to be involved in binding, although the mechanisms are largely unknown; and (iv) Ser and sometimes Thr residues, in or around the cationic clusters, are usually essential for transport and have been implicated as phosphorylation sites [102-105]. The apparent role of phosphorylation in binding, translocation or release of the transported macromolecule remains to be elucidated.

At present, a large number of monopartite [104,105•,106-115] and bipartite [116-120] NLS sequences have already been identified, and representative sequences are listed in Table 2. The number of routes for nuclear uptake and the extent to which multiple pathways utilize common mechanisms is unknown. The first and most well-characterized example of a monopartite NLS is derived from the large tumor antigen of simian virus 40 (SV40) [104,105•]. A seven amino acid sequence (^{126}Pro-Lys-Lys-Lys-Arg-Lys-Val^{132}) is the minimum sequence that is sufficient to confer nuclear transport. A single substitution of Asn or Thr for Lys^{128} abolishes nuclear targeting, while mutation of the other basic residues diminishes the ability to transport proteins across the nucleus [106]. When a peptide containing this NLS sequence was non-covalently associated with plasmid and injected into the cytoplasm of zebrafish embryos, nuclear uptake of the plasmid was 50- to 100-fold faster than the control plasmid containing reversed NLS sequence [106]. This NLS sequence is believed to be involved in ionic interactions with a cluster of anionic amino acid residues either in the transporter receptors or in the nuclear pore [106]. Coincident with the identification for SV40 large T antigen NLS, a putative NLS sequence (Asn-Lys-Ile-Pro-Ile-Lys-Asp) was also identified in the amino terminus of yeast α-2 mating factor, which was shown to enhance nuclear accumulation of β-galactosidase [108].

Despite the large number of putative single cluster NLS sequences identified to date, there is hardly any structural or physicochemical characterization either of an NLS sequence by itself, or bound to any of the receptor proteins. The absence of an absolute consensus in the primary sequence suggests that slightly different NLS sequences may adopt a common secondary structure or motif. The positively charged side chains of Lys and Arg in short NLS sequences are expected to repel each other and favor a random coil structure, as observed for K_a [GeneMedicine, unpublished results]. However, the apparent absolute requirement for Lys at position 128 and to some extent Lys/Arg at positions 129 and 131 of the SV40 large T antigen NLS (or their equivalent positions in the other NLS sequences), suggest that upon interaction of these NLS sequences with their cytoplasmic receptors, or possibly proteins in the NPC, they adopt a secondary motif where the residues at these positions are structurally invariable in space. A compilation of 12 single cluster NLS sequences showed an apparent sequence consensus as $X^1X^2Lys^3(Lys/Arg)^4X^5(Lys/Arg)^6$. Molecular modeling of the six amino acid residues in the NLS cluster showed that, despite the wide variability in the amino acid composition at positions 1, 2 and 5 and the basic amino acid residues at positions 4 and 6, they maintained very similar spatial orientations (Figure 2). Only minimal structural adjustments may be required for all of them to adopt the same secondary structure. This idea is consistent with the observation that NLS peptide antibodies recognize different nuclear proteins [99].

The bipartite NLS sequences, often found in mammalian nucleoproteins, generally consist of two clusters of cationic residues separated by a spacer of 10 to 12 residues [$(K/R)_{2-3}$-$(X)_{10-12}$-$(K/R)_{3-5}$]. The first of the bipartite NLS sequences that

experimentally demonstrated nuclear targeting was nucleoplasmin [116], with the minimal sequence of Lys-Arg-Pro-Ala-Ala-Ile-Lys-Lys-Ala-Gly-Gln-Ala-Lys-Lys-Lys-Lys (Table 2). The length of the spacer may be variable up to 20 residues, and sometimes contain amino acid residues of specific charges. It has been suggested that the bipartite clustering of cationic residues improves binding of the NLS to the transport receptors, but data to substantiate this hypothesis is limited.

There are at least two general strategies for assessing the enhancement of nuclear transport by NLS peptides. The first involves electrostatic binding of plasmid to cationic NLS-containing proteins [121,122] or peptides [123,124] or lipids [125,126] by direct mixing. For example, cells transfected with plasmid/lipofectin complexes expressed luciferase and β-galactosidase 20- and 2.5-fold higher, respectively when a chimeric NLS-containing histone-derived protein was incorporated into the complexes [127]. Similarly, nuclear uptake of plasmid was reported to be 50- to 100-fold more rapid when Cys-Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val-Gly was mixed with plasmid and injected into zebrafish embryos [126]. When a similar peptide (Pro-Lys-Lys-Lys-Arg-Lys-Val-Lys-Lys-Lys-Lys) was included in the formulation of lipid/plasmid complexes, a modest enhancement in gene expression was also observed [125].

The second strategy involves direct covalent attachment of the NLS peptide to the plasmid. For example, covalent attachment of Cys-Gly-Tyr-Pro-Lys-Lys-Lys-Arg-Lys-Val-Gly-Gly to double-stranded DNA [128] and Cys-Gly-Tyr-Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val-Gly-Gly to plasmid both induced nuclear accumulation of fluorescently labeled DNA in digitonin-permeabilized cells. The mechanism of transport is believed to follow the classical NPC-mediated pathway because transport can be inhibited by, for example, NLS-BSA conjugates [120]. However, microinjection of this plasmid containing greater than one NLS per 25 base pairs of plasmid did not lead to gene expression. However, Zanta *et al* [129••] demonstrated a single NLS peptide covalently linked to one end of *CMV-luciferase* gene can enhance *in vitro* transfection by 10- to 1000-fold irrespective of the cationic carriers or the cell types used. Zanta hypothesized that the NLS docks the DNA onto the nuclear pore complex and the 3 nm wide DNA present in the cytoplasm is then translocated through a nuclear pore. As DNA enters the nucleus, it is quickly condensed into a chromatin-like structure, which provides a mechanism for threading the remaining worm-like molecule through the pore. In this proposed mechanism, a single NLS peptide located at the end of the plasmid would be the most efficient structural presentation. Furthermore, it was postulated that multiple signals on the same plasmid might even inhibit nuclear transport if two or more of the NLS signals are simultaneously docked to different neighboring nuclear pore complexes.

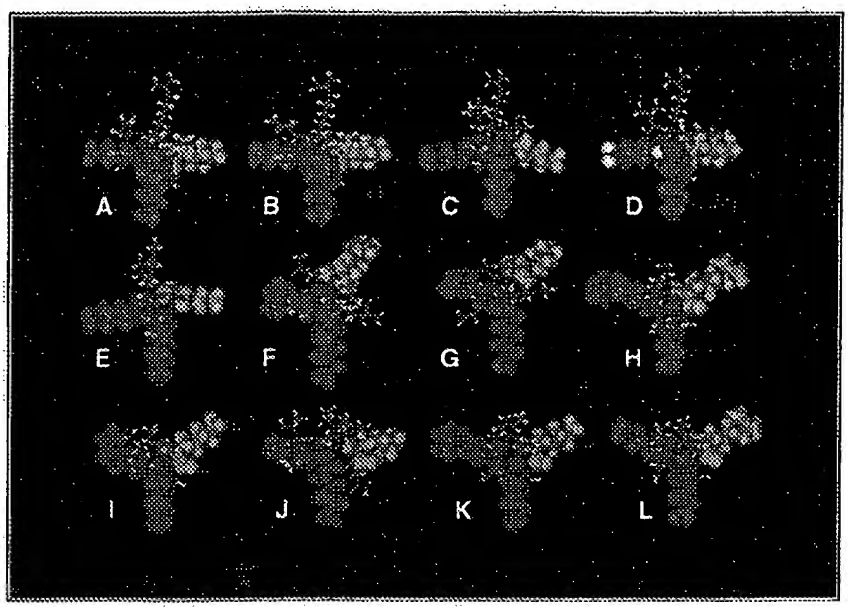
Table 2. Typical nuclear localization signal (NLS) sequences.

Nuclear Proteins	Minimal NLS[1]	Number of amino acids	Position of signal[2]	References
Single cluster NLS sequences				
SV40 large T-antigen	PKKKRKV	708	126-132	Lanford and Butel [105]
Yeast mat α2	NKIPIKD	210	1-13	Hall *et al* [109]
SV40 VP 2/3	PNKKKRK	289	317-323	Gharkhanian *et al* [110]
Adenovirus E1A	SCKRPRP	289	285-289	Lyons *et al* [111]
Yeast ribosomal L29	KTRKHRG KHRKHPG	148	6-12 23-29	Underwood and Fried [112]
Yeast histone H2B	GKKRSKA	130	28-33	Moreland *et al* [113]
HIV *rev*	RRNRRRRW	116	38-45	Malim *et al* [114]
Human lamin A	SVTKKRKLE	702	408-444	Loewinger and McKeon [115]
Rat glucocorticoid-R	RKTKKKIK	795	497-524	Picard and Yamamoto [116]
Human c-*myc*	PAAKRVKL RQRRNELKRSF	493	320-328 364-374	Dang and Lee [117]
Bipartite NLS sequences				
Xenopus nucleoplasmin	KRPAATKKAGQAKKKKL	200	155-171	Robbins *et al* [118]
Xenopus N1/N2	LVRKKRKTEEESPLKDKDAKKSKQ	589	530-553	Kleinschmidt and Seiter [119]
Human SRY	KRPMNAFIVWSRDQRRK		61-77	Poulat *et al* [120]
Mouse FGF3	RLRRDAGGRGGVYEHLLGGAPRRRK		52-76	Kiefer *et al* [121]
Poly(ADP-ribose)polymerase	KRKGDEVDGVDEVAKKKSKK		207-226	Schreiber *et al* [122]

[1]NLS sequenced from a large region are usually chosen based on homology with another NLS and therefore do not necessarily present an actual NLS. An indicated sequence may represent more than or only a part of a signal.

[2]Position of signal refers to the position of the amin acids in the sequence of native, NLS-containing protein.

Figure 2. Molecular modeling of the common structural motif of different putative single cluster NLS sequences.



A glycine residue was included before and after the putative NLS sequence in order to provide an extended backbone for potential hydrogen bonding with the first or last amino acid in the NLS sequence. The sequences modeled are from the following proteins: (A) SV40 large T antigen, Pro-Lys-Lys-Lys-Arg-Lys; (B) SV40 VP2, Asn-Lys-Lys-Lys-Arg-Lys; (C) Lamin A, Val-Thr-Lys-Lys-Arg-Lys; (D) N1 protein site II, Val-Arg-Lys-Lys-Arg-Lys; (E) Nucleoplasmin, Gln-Ala-Lys-Lys-Lys-Lys; (F) Polyomavirus large T antigen site II, Ser-Arg-Lys-Arg-Pro-Arg; (G) Histone H2B, Gly-Lys-Lys-Arg-Ser-Lys; (H) Adenovirus E1A, Ser-Cys-Lys-Arg-Pro-Arg; (I) N1 protein site I, Asp-Ala-Lys-Lys-Ser-Lys; (J) Rat glucocorticoid receptor, Thr-Lys-Lys-Lys-Ile-Lys; (K) Human c-*myc*, Ala-Ala-Lys-Arg-Val-Lys; and (L) Polyomavirus large T antigen, Pro-Pro-Lys-Lys-Ala-Arg. Based on the apparent consensus sequence $X^1X^2K^3(Lys/Arg)^5X^6(Lys/Arg)^8$, the positively charged amino acid side chains at positions 3, 4 and 6 are shown as space filling models pointed down, to the left, and to the right, respectively. The rest of the residues are shown in ball-and-stick rendition. Each structure is shown approximately along the direction of chain propagation, with the N-terminus facing the reader and the C-terminus going down into the paper. The structures are presented in a manner that all three critical residues have approximately similar spatial orientations. Note that sequences in A-E also have positively charged residue at position 5, all of which are extended upward. Although the peptide backbones are not shown, some of them have very different in minimized structures. Molecular modeling was performed on MS1 Insight II Software (Molecular Simulation Inc, San Diego, CA) by constructing an α-helical form of all peptides as starting points and allowing 10000 iterations of a conjugate gradient minimization or until the RMS derivative went down to zero.

Self assembly of peptide-based delivery components

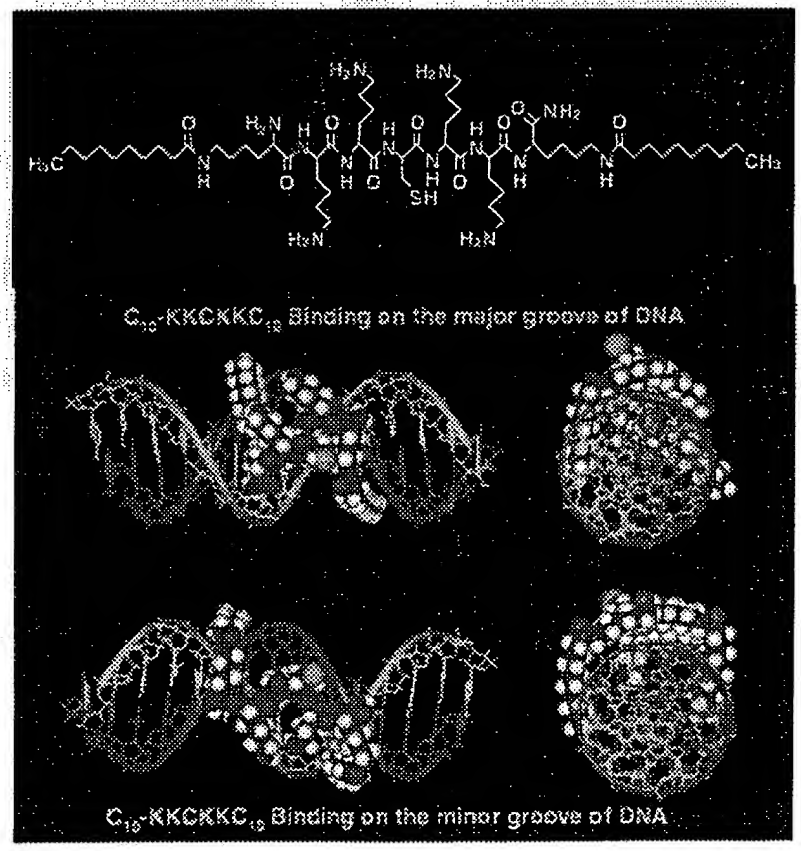
Identification of the major components of a peptide-based gene delivery system represents only part of the overall task of addressing the various limitations that are expected to be encountered, from the site of administration of the plasmid to the nucleus of the target cell. Another major challenge is to design these components to enable their systematic self-assembly and sequential release within the cell compartments. In principle, assembly should start with DNA condensation. Conversely, based on their predetermined functions, the order of release of peptide components should be from the targeting peptide → lytic peptide → condensing peptides → NLS peptide. However, in practice, the difficulty in achieving this order is such that the investigator often measures only gene expression.

As shown schematically in Figure 1, a peptide-based gene delivery system can include a number of essential engineering or assembly features for functional optimization: (i) the ability to conjugate each peptide moiety to the same or different DNA binding/condensing peptide 'templates'; (ii) the flexibility in controlling the affinity of DNA binding template; (iii) the ability to control the type and density of each peptide moiety; and (iv) the flexibility to control the type and length of linkers necessary for the functionality of each moiety.

Since the key feature of the approach is the DNA binding/condensing peptide template, structure-based control of its affinity for DNA is important. It is generally known that the affinity of the condensing peptide for DNA can be controlled based on the number and density of the polycationic cluster [39•], but its major limitation is the instability of purely electrostatic interactions in the presence of salt or serum. Therefore, it is necessary to include functional groups that improve the stability of the DNA binding template complex in salt. One approach is the inclusion of hydrophobic functional groups in the polycationic template. Our molecular modeling studies indicate that decanoyl chains attached to both ends of a short polylysine can fit well into the relatively hydrophobic major groove, as well as the minor groove of DNA (Figure 3). The strength of hydrophobic interaction can be controlled by the length of the acyl chains. A variation to this approach was where acyl groups were attached to a cationic head group containing a sulfhydryl functionality, and the two peptides after complex formation with DNA were crosslinked [31•]. This approach produced 23 nm particles that remained stable over several days. Furthermore, DNA binding and condensation can be improved by the addition of Trp in the polycationic sequence, possibly due to both increase in hydrophobicity and the ability of the indole ring to intercalate into the double-stranded DNA [35•,36,145••]. These peptides are known to bind DNA much tighter compared to their corresponding unmodified polylysines.

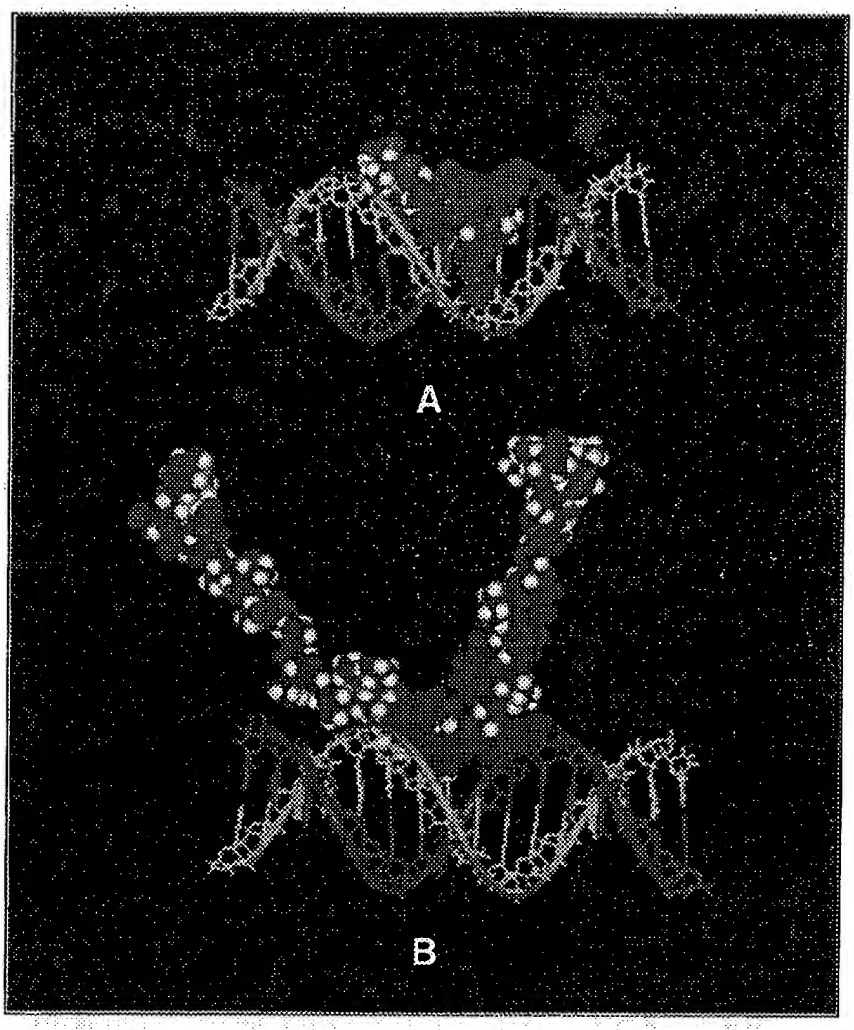
Based on the predicted sequence of release, the NLS peptide has to have the highest affinity for the DNA relative to the targeting and the fusogenic peptides. Although covalent attachment of NLS peptide to DNA has been successful in demonstrating nuclear transport [128,129••], non-covalent attachment of the NLS to the DNA by electrostatic interaction or alternatively covalent attachment of the NLS to a DNA binding peptide template, would be preferred in order to allow total release of the DNA from the NLS peptide during or right after nucleoplasmic transport. For example, NLS peptides have been conjugated to sequence specific peptide nucleic acid (PNA) clamps to demonstrate nuclear transport of DNA [201]. Another variation of this approach was the use of a chimeric protein containing a DNA-binding domain and an NLS domain [130•,131]. However, the general approach of covalently attaching an NLS peptide to a DNA binding molecule should be designed with care because, being highly positively charged, the NLS peptide would be expected to bind to the plasmid (Figure 4A), condense it, and compete with the condensing peptide for binding to the DNA and may make the NLS less available for binding to the cytoplasmic or NPC receptor. Consistent with this idea is the report [132] that the NLS receptor in the NPC is mutually exclusive for DNA and for the NLS ligand. One possible way to circumvent this problem is to use neutral or anionic NLS sequences [133] or extend the amino acid sequence of the NLS to include negatively charged residues upstream and downstream of the NLS sequence [104] to make the net charge of the peptide close to neutrality. Molecular modeling (Figure 4B) of the long NLS sequence of the SV40 T-antigen [103] (Cys-Tyr-Asp-Asp-Glu-Ala-Thr-Ala-Asp-Ser-Gln-His-Ser-Thr-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Pro-Lys-Asp-Phe-Asp-Ser-Glu-Leu-Leu-Ser) show that the negatively charged upstream and downstream residues of the long NLS sequence are repelled by the negatively charged phosphate backbone. This electrostatic repulsion would be expected to significantly destabilize NLS binding to the DNA and make it available for binding to the NLS receptor proteins.

Figure 3. Molecular modeling of a diacylated condensing peptide, decanoyl-Lys-Lys-Cys-Lys-Lys-decanoate.



The chemical structure is shown on top, and its space filling models after docking and minimization onto the major groove (middle) and th minor groove (bottom) of DNA. Based on DNA chain orientation, the left models are viewed sidewise from the top, while the right models are cross-section diagrams. The two DNA backbones are shown as dark and light solid ribbons, and the nucleotide bases are rendered as lines. The hydrogen atoms of the peptide are shown in white while the rest of the atoms are shown as dark spheres. In both cases the sulfhydryl group (where sulfur is shown as the largest gray sphere) is projected outside and favorable to conjugation with other peptide ligands. In both the major groove and the minor grooves, the models show that the decanoyl groups bend inward and appear to 'hug' the DNA strands. Molecular modeling was performed by manually docking the α-helical form of the diacylated condensing peptide onto the respective grooves of DNA and performing 10,000 iterations of a stepwise conjugate gradient minimization or until the RMS derivative went down to zero.

Figure 4. Space filling molecular m deling of the interaction of two f rms f the SV40 larg T antigen NLS peptides with DNA.



(A) The putative Pro-Lys-Lys-Lys-Arg-Lys NLS sequence with an extra Gly in both N- and C-termini to provide backbone amide bonds for potential hydrogen bonding.

(B) The NLS sequence extended upstream and downstream to include the negatively charged residues, amino-Cys-Tyr-Asp-Asp-Glu-Ala-Thr-Ala-Asp-Ser-Gln-His-Ser-Thr-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Pro-Lys-Asp-Phe-Glu-Ser-Glu-Leu-Leu-Ser-amide. Each peptide was manually docked onto the major groove of DNA and 10000 iterations of stepwise conjugate minimization were performed until the RMS derivative went down to zero. The hydrogen atoms in the peptides are shown in white, while the rest of the atoms are shown in dark gray.

Endosomolytic peptides have usually been incorporated into the condensed plasmid complex by non-covalent association with the condensing peptide. This simple self-association is facilitated by electrostatic interaction between the usually negatively charged fusogenic peptide and the positively charged condensing peptide, as seen in the case of K_8 and JTS-1 [18••,145••]. The plasmid/K_8 complex does not transfect cells *in vitro*, but addition of the lytic peptide JTS-1 showed dose-dependent increase in transfection efficiencies. Unfortunately, the stability of that formulation is very much dependent on salt concentration, changes in pH, and even the sequence and manner of addition of the components. This instability is likely the result of DNA and JTS-1 competing for the positively charged condensing peptide, K_8. An improvement of this system was achieved by attaching two palmitoyl groups to both the condensing peptide and the fusogenic peptide to produce the corresponding lipophilic lytic peptide (Pam_2-Lyt) and lipophilic condensing peptide (Pam_2-K_8) [200]. This modification was designed to promote association of the lytic and the condensing peptides through their dipalmitoyl groups, which is less susceptible to ionic strength. Formulations containing these lipophilic peptides produced small particles (80 nm) that were more stable to salt and serum challenge [Valentis Inc, unpublished results]. A similar modification, but with only one palmitoyl group attached to the Ser-Pro-Lys-Arg-Ser-Pro-Lys-Arg-Ser-Pro-Lys-Arg condensing peptide also produced improved transgene expression efficiency *in vitro* [90].

An alternative approach incorporates the DNA condensing and lytic properties into one peptide. The amphipathic cyclic decapeptide, Gramicidin S, has an ability to condense plasmid as well as the property to destabilize membrane

[137]. The two functional domains, the positively charged ornithine residues and the hydrophobic amino acids, are on opposite faces of the cyclic peptide. Gramicidin S interacts with plasmid at a 1:1 (-/+) charge ratio, leading to efficient transfection when dioleoylphosphatidylethanolamine (DOPE) is added at a lipid/peptide molar ratio of 5:1. However, the limited flexibility in altering the structures of these cyclic peptides has greatly restricted the ability to make analogs with efficient DNA binding and membrane destabilization properties. As an alternative strategy, Wyman *et al* [134] synthesized a cationic amphipathic peptide KALA (repeats of Lys-Ala-Leu-Ala) to serve for both DNA binding and membrane destabilization. KALA was reported to undergo a pH-dependent random coil to amphipathic α-helical conformational change as the pH is decreased from 7.5 to 5.0. One face of KALA displays hydrophobic leucine residues, and the opposite face displays hydrophilic lysine residues. KALA binds to plasmids and, at 10/1 (+/-) charge ratio, mediates transfection of a variety of cell lines. KALA sequence provides an interesting starting point for a family of peptides that can be used as a template to incorporate other functions to improve plasmid delivery. For example, a Cys residue can be incorporated into a KALA analog to act as a conjugation site for cell targeting ligands or NLS peptides.

Various plasmid formulations containing the above components, ie, condensing peptide, NLS and lytic peptide are effective for *in vitro* transfection and, to some extent, *in vivo* gene delivery. It is even possible that a particular peptide/DNA formulation may passively target specific organs. For cell-specific targeting, ligands must be incorporated into the formulation complex, either by covalent binding or through non-covalent interactions. Traditionally, targeting ligands such as galactose [65], mannose [67], transferrin [41] and folate [46], have been covalently conjugated to the condensing carrier, such as polylysine. However, polylysines strongly bind to DNA and may actually retard the release of the targeting ligand from the DNA carrier complex. Therefore, the affinity of the cell-targeting template to the formulation complex must be either ≤ that of the lytic peptide to facilitate early release relative to the template-bound NLS.

Even with structure-based design, self-assembly of gene delivery components remains a big challenge because complex formation is affected by a variety of media constituents and conditions. In an attempt to simplify complex formation, an elegant approach was introduced by Ledley and Stankovics [147•] where multiple gene delivery elements were designed into a single, chimeric protein. This strategy of incorporating the DNA-binding, cell targeting, lytic and NLS components into a multi domain protein that can be produced recombinantly, or even synthetically, has recently generated a lot of interest. For example, a recombinant fusion peptide containing a Gal4 DNA-binding domain was linked to a fragment of the invasin protein of *Yersinia pseudotuberculosis* [136]. Invasin recognizes integrin receptors on the cell surface and enables the pathogen to penetrate the cells by a non-phagocytic process. Gal4 is a transcriptional activator that recognizes a specific 17 bp DNA sequence. The plasmid was constructed to include eight copies of the Gal4 binding sites. A plasmid/peptide complex, prepared at a ratio of fusion peptide to DNA ratio at which all the Gal4 recognition sites would be occupied, gave significant targeted gene transfer *in vitro*. Transfection was dependent on the extent of DNA condensation and on the presence of the Gal4 binding sequences on the plasmid. Transfection was reduced in the presence of anti-integrin receptor antibody, suggesting plasmid uptake via a receptor-mediated process. Recently, Chan *et al* [132] confirmed that the amino acid terminal domain of Gal4 has the ability to both bind to a specific 17 bp DNA sequence and confer nuclear localization of the large (> 476 kDa) protein. Interestingly, the NLS of Gal4 is distinct from conventional NLS sequences, as it is recognized exclusively by the nuclear pore targeting β-subunit of the NLS receptor importing complex, rather than the α-subunit.

After assembly of the peptide components into a plasmid delivery complex, the overall surface charge of formulations containing the four major components discussed above will depend largely on the sum of the electrostatic charges of the individual components and their arrangement within the complex. Positively charged formulations may interact with plasma proteins and cell surfaces in a non-specific manner, thus reducing the ability to actively target a complex to a specific site, especially after systemic administration. Even negative charged formulations can sometimes bind to specific serum proteins, preventing them from reaching the target sites. In an attempt to overcome this problem, steric components have been incorporated into the formulation to shield the surface charge of the formulation from non-specific interaction with serum components as well as surfaces of non-targeted cells. The most commonly used polymer for steric protection is linear polyethylene glycol (PEG) of various chain lengths [137]. Although there have been numerous reports about the effects of the chain length of PEG, there has been no consistency either in the results or in their interpretations. The formulation mixture is often already too complicated to interpret the results, even in the absence of the steric components. It is not even clear what, if any, is the relationship between the chain length of PEG and its chain density. There will be a need for further research to determine the extent of protection afforded by each PEG chain of a specific length.

The use of steric molecules with more defined structure, or at least more amenable to structural characterization, has not been explored yet. One logical approach is to use short, branched molecules, such as an acetylated multiple antigenic peptide (MAP) system containing a branched polylysine core [138]. The surface area/molecule can be controlled by the extent of branching steps, while the space or distance between the steric molecule and the formulation complex can be controlled by the length of linker. These branched polylysine derivatives can be anchored covalently or non-covalently either to the condensing, the lytic or the targeting peptides. In either case, the amount of steric protection has to be optimized to maintain proper exposure and function of these peptides.

Peptides in combination with oth r delivery systems

While peptides can be structurally engineered to perform specific functions in a gene delivery complex, peptides have also been successfully incorporated in other gene delivery systems, such as lipid- and polymer-based systems in order

to enhance their gene transfer efficiencies. Since these non-peptide-based delivery systems are discussed elsewhere in this issue, only the role of peptides in the context of lipid- and polymer-based systems will be briefly presented here.

The entrapment efficiency of plasmid within anionic or neutral liposomes is generally low because of the large dimension of plasmid compared to the internal diameter of the vesicles [139]. To enhance the encapsulation efficiency, the plasmid may be pre-condensed with short positively charged molecules, such as peptides and then encapsulated into liposomes. Peptides such as protamines have recently been used in combination with cationic lipid-mediated gene delivery systems and enhanced gene transfer [140].

Peptides can also be used for cell-specific targeting of lipid/plasmid formulations. One approach is to add hydrophobic moieties into the targeting peptide ligand, such as acyl groups, that can promote insertion into the lipid membrane layer. The hydrophobic functionality of the targeting peptide can also be selected to promote simple adhesion onto the surface of liposomes. For example, incorporation of Gramicidin S and plasmid into asialofetuin-labeled liposomes has enabled receptor-mediated gene delivery into primary hepatocytes [141].

Another approach has been to incorporate a peptide that has both DNA binding ability and membrane disrupting activity into a liposome or a lipid/plasmid complex. This approach was illustrated by chemical coupling of a membrane disrupting cationic peptide, mellitin, with the fusogenic lipid DOPE [142]. The resulting molecule, dioleoylmelittin, was able to complex plasmid and mediate efficient gene transfer, which was even enhanced in the presence of serum. In another example, the cyclic cationic amphipathic decapeptide, Gramicidin S, was included into a DOPE lipid/DNA composition, and facilitated plasmid delivery *in vitro* [143]. Both the peptide and the phospholipid component were required for efficient gene transfer. Peptides with fusogenic activities can also be incorporated into lipid formulations to enhance endosomolysis. For example, incorporation of influenza virus-derived peptides into positively charged 1,2-dioctadecylamidoglycylspermine (DOGS)/plasmid complex enhanced gene expression by 3- to 30-fold [78]. However, transfection of more electroneutral formulations is strongly increased by peptides up to 1000-fold, indicating that for these particles endosomal escape is a rate-limiting step. In another example, when the fusogenic peptide GALA was non-covalently associated with luciferase plasmid/DOTAP:DOPE [2/1 (+/-)] complexes, the level of luciferase expression in HeLa cells increased by 4-fold [144].

Synthetic peptides have also been used for enhancing transfection efficiencies of polymer-based systems. For example, when JTS-1 and its analog dipalmitoylated at the carboxyl-terminus were non-covalently incorporated into chitosan/plasmid formulations, transfection of COS-1 cells was enhanced 4-fold [11]. Similarly, polyethyleneimine/DNA complexes containing INF-type lytic peptides had up to 10-fold increase in transfection [78]. A variation of this approach was the use of polylysine to condense plasmid prior to its entrapment into poly(D,L-lactide-co-glycolide) microspheres [145]. Results showed that the entrapped plasmid was released from the microspheres over several days. Although no transfection data were presented, this approach can be potentially used for sustained gene delivery and expression.

In summary, the peptides can be structurally engineered to impart very specific functions that the lipid and the polymer systems cannot provide efficiently, most notably in the areas of cell-specific targeting, endosome lysis and nuclear localization.

Perspectives

A challenge for plasmid-based gene therapy is to surmount the key limiting steps from the site of administration to the nucleus of the target cell, such as cell-specific targeting, endosomal release, cytoplasmic transport and nuclear uptake and retention of plasmids. Synthetic peptide-based gene delivery systems allow rational design and systematic evaluation of specific functional motifs. With synthetic peptides it should be possible to package the plasmid into a very small particle, deliver the plasmid complex into specific targets, enhance cellular uptake, endosomal release and dissociation of the plasmid from the complex into the cytoplasm, transport the plasmid to the nucleus and protect it from nuclease degradation during transit. The development of peptide-based gene delivery systems is still relatively in its early stage, but rapid progress in devising delivery systems based on rational design can be expected.

Ironically, the key feature of any gene delivery system, peptide-based or otherwise, which is most difficult to control, is a well-defined time- and space-controlled release of the functional moieties (or uncoating of the plasmid) after they have rendered their functions. Ideally, the plasmid has to be completely free of any delivery component upon reaching the nucleus. Conversely, prematurely uncoating the plasmid results in plasmid degradation.

There are important areas of research on peptide-based gene delivery systems that clearly need to be pursued. One area is in the utilization of endosomal acidification to design peptides that are not only for pH-dependent membrane activity, as clearly seen in the INF family of peptides, but also for the controlled release of the targeting peptide and probably most of the condensing peptides. Secondly, a strategy that is beginning to emerge, but still needs further development, is the use of multifunctional peptides or chimeric proteins. These peptides can be in the form of a single peptide performing two functions, as in the case of the DNA binding ability and membrane activities of the KALA and the H5WYG peptides, or one peptide with two distinct functional domains, as in the case of the DNA binding and membrane active Gal4-invasin fusion peptide. Thirdly, the issue of incorporating non-linear peptide-based steric groups to protect the plasmid formulation during systemic transit also needs to be explored, such as the MAP-type of peptide structures. Fourthly, the feasibility of using peptide/liposomes or lipopeptides should be further investigated. Substituting the polar heads of the cationic lipids with very short cationic peptides offer, for instance, a tremendous design flexibility to suit specific DNA binding sites.

In conclusion, synthetic peptides offer a great potential for the development of efficient gene delivery systems. The technology is still relatively new, but novel and exciting approaches are now beginning to emerge based on empirical and experimental considerations.

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Application of membrane-active peptides for drug and gene delivery across cellular membranes

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Abstract

Naturally occurring peptides and protein domains with amphipathic sequences play a dominant role in physiological, lipid membrane-reorganizing processes like fusion, disruption, or pore formation. More recently this capacity to modulate membrane integrity has been exploited for drug delivery into cells. Incorporation of synthetic membrane-active peptides into delivery systems has been found to enhance intracellular delivery of drugs including oligonucleotides, peptides, or plasmid DNA. In the majority of applications, the amphipathic peptides are designed to act after uptake by endocytosis, releasing the delivered agent from intracellular vesicles to the cytoplasm. Alternatively, peptides might mediate direct drug transfer across the plasma membrane. Although encouraging results have been obtained with the use of synthetic peptides to enhance cellular delivery of various compounds, the naturally evolved mechanisms observed in the entry of viruses or protein toxins are still far more efficient. For the development of improved synthetic peptides and carrier systems a better understanding of the molecular details of membrane-destabilization and reorganization will be essential.

Keywords: Endosome disruption; Membrane fusion; DNA transfection; Amphipathic peptides; Cytosolic delivery

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Abbreviations: Bti, *Bacillus thuringiensis israelensis*; CTL, cytotoxic T lymphocyte; DT, diphtheria toxin; HA, hemagglutinin; HIV, human immunodeficiency virus; HRV, human rhinovirus; PE, *Pseudomonas* exotoxin; SIV, simian immunodeficiency virus

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1. Introduction

Many therapeutic agents (drugs, protein, liposomes, genes) act at intracellular sites and depend on an efficient delivery into the target cell. Several

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low-molecular weight compounds are readily taken up by passive diffusion processes through the cell membrane. Charged and large compounds cannot penetrate through lipid membranes and require active transport mechanisms for uptake into cells. The efficient cellular process of receptor-mediated endocytosis can be utilized by physical or molecular linkage of the compound to a domain that can bind a cell surface receptor (e.g., for review see Ref. [1]). After uptake into endosomes or other internal vesicles, the material is topologically still outside the cell, with a membrane separating it from the cytoplasm. Therefore, with or without endocytosis, delivery across cellular membranes is required. For this step several processes may be considered: (i) direct transfer through cell surface membrane by lipid membrane fusion (e.g. with liposomes, virosomes) or transient permeabilization of the cell membrane (for example by electroporation, not a topic of this paper); (ii) alternatively, after endocytosis, transfer across vesicular membranes by lipid disruption, pore formation, or fusion. Several of these membrane reorganization processes are involved in the entry of viruses or microorganisms, and are also triggered by protein toxins and defense peptides. Related processes are important in biological events like the intracellular vesicle budding and fusion, or fusion of cells [2,3], sperm–egg fusion [4,5], or the immune response [6–9].

Although the individual steps of membrane reorganisation are physically not well-understood, the membrane destabilizing element often can be assigned to a short peptide domain of about 20–30 amino acids. This paper surveys several classes of these protein domains or peptides and the current hypotheses on their action. The possible impact of such membrane-destabilizing domains on intracellular drug delivery is reviewed. Ideally, small synthetic peptides should serve as membrane-active part of the drug delivery vehicle. The use of membrane-active peptides for the delivery of other agents such as small or medium-size chemical compounds, peptides, oligonucleotides, or proteins is reviewed. We discuss in more detail the capacity of synthetic peptides derived from influenza virus and related sequences to disrupt lipid membranes of liposomes, erythrocytes, or endosomes of living cells. The concept of using these peptides for endosome disruption has been found important for efficient gene delivery of polycation-complexed nucleic acids [10,11].

2. Enhancement of cytosolic delivery by viruses

In the viral infection process, membrane-active protein domains (Table 1) mediate the transfer of the viral genome into the cytoplasm of the cell. For some viruses (like Sendai virus) the close contact of viral membrane and the cell membrane upon binding to cell surface receptors is sufficient for the viral fusion domains to induce membrane fusion, releasing the nucleocapsid into the cytoplasm. Other viruses

Table 1
Membrane-active elements of viruses

Virus	Membrane-active protein	Fusion/disruption	Site of action/pH properties
Enveloped viruses			
Influenza virus	Hemagglutinin subunit HA-2	Fusion	Endosome/acidic pH
Semliki forest virus	E1		
Vesicular stomatitis virus	G		
Vaccinia virus	14-kDa protein		Cell surface/neutral pH
Sendai virus	F1		
Measles virus	F1		
HIV	gp41		
SIV	gp32		
Naked viruses			
Adenovirus	Unknown (penton base?)	Vesicle disruption	Endosome/acidic pH
Rhinovirus	vp1	Pore formation	
Polio virus	vp1		
Coxsackie virus	vp1		

such as adenovirus, influenza virus or the picornaviruses are internalized by receptor-mediated endocytosis. Here the endosomal acidification process activates the protein domains that trigger the fusion of the viral membrane and endosomal membranes in the case of enveloped viruses, or pore formation and/or disruption of the endosomal membrane in the case of non-enveloped viruses. In several viruses amphipathic peptide sequences have been identified that are involved in the membrane-destabilization process (see Section 4 of this review).

The viral entry has been found to influence the intracellular delivery of other macromolecules [12–15]. The presence of adenoviruses during receptor-mediated uptake of toxin conjugates enhances the delivery of the conjugates to the cytoplasm [16–18]. Also, gene transfer mediated by the efficient receptor-mediated uptake of transferrin–polylysine-coated plasmid DNA [19,20] is strongly enhanced by addition of replication-defective adenovirus particles [21,22]. Co-internalization of both adenovirus and the DNA complex into the same endosome is essential for the release of the DNA particles into the cell cytoplasm; linkage of the adenovirus to the DNA/ligand–polylysine complex [23–28] further improves the gene transfer efficiency and is required for delivery into cell types with limited entry of adenovirus (e.g. K562 cells, T or B cell lines). Because only the endosome disrupting activity of the viral capsid is required, it is possible to inactivate the viral genome by treatment with methoxypsoralen plus irradiation [22,29].

For release of transferrin-coated DNA complexes from endosomes, other viruses have been identified as alternative to the human adenoviruses, like the CELO virus, a chicken adenovirus which displays both an endosome-disruptive and a gene transfer-enhancing activity very similar to the human virus [30]. Addition or linkage of rhinovirus particles to the DNA complex showed a similar, but slightly less pronounced effect [31]. This different activity is in line with the finding that adenovirus and rhinovirus show different behaviour concerning the release of endosomal content [32].

In a different approach, virosomes, i.e. liposome-like reconstituted envelopes of viruses loaded with the agent to be delivered, are used to exploit the efficient delivery machinery of enveloped viruses for drug delivery. Such systems based on influenza virus (delivery via fusion with the endosomal membrane after endocytosis) or Sendai virus (delivery via fusion with the cell membrane) and related approaches have been found effective for the delivery of drugs, proteins or genes [33–37]. Virosomes have already been applied in humans for the purpose of vaccination [38,39]. More detailed discussions of virosomes can be found in the references listed above.

3. Enhancement of cytosolic delivery by exotoxins and other bacterial proteins

The activity of many protein toxins depends on an efficient mechanism for the delivery of the toxic domain to the cytosol. Exotoxins like diphtheria toxin (DT) or *Pseudomonas* exotoxin (PE) consist of two polypeptide chains. The 'A' chain contains the toxic activity which inhibits essential cellular steps in the cytosol (like protein synthesis), and the 'B' chain contains functions that trigger uptake into cellular vesicles and translocation of the toxin 'A' chain from endosomes to the cytosol [40,41]. For example, upon exposure to low pH the B fragment of DT interacts with the endosomal membrane. Part of its N-terminal T (transmembrane) domain is responsible for the formation of cation-selective channels; a 61-amino acid region of the protein (including α-helices TH8 and TH9) has been found sufficient to form channels with the same pH-dependent properties as that of the whole toxin [42–45]. Another amphipathic α-helix in the N-terminal part of fragment B, TH1, efficiently promotes translocation of fragment A, but does not interact with the hydrophobic part of the membrane phospholipids [46].

This efficient translocation process has been exploited for targeted killing of cells presenting specific surface molecules ('immunotoxin approach', reviewed in Refs. [47,48]). For example, recombinant immunotoxins have been generated [49] containing truncated PE or DT toxins (defective in their cell-binding domain) linked to single-chain versions of antibody domains directed to specific cell-surface molecules. Also translocation of other biological active proteins into cells, like acidic fibroblast

growth factor has been achieved by fusion to diphtheria toxin [50].

The cytolysins staphylococcal α-toxin, streptolysin O, or listeriolysin [51] belong to a class of bacterial proteins that lyse cells (or cellular vacuoles) by formation of large aqueous pores. The process requires multimerization of the proteins within the membrane; in several cases it requires binding to specific lipids (such as cholesterol). The family of sulfhydryl-activated hemolysins (activity enhanced by reducing agents, suppressed by oxidation) includes listeriolysin O or streptolysin O. Listeriolysin O is the only member where activity is dependent on low, endosomal pH [52,53], which enables growth and spread of the intracellular bacterial parasite *Listeria monocytogenes*.

Streptolysin O and staphylococcal α-toxin have been used as standard reagents for intracellular delivery of reagents by permeabilization of the cells [54]. Antisense oligonucleotides were delivered into cells without any apparent cytotoxicity [55]. Recently, a related cytolysin, perfringolysin O, was used to efficiently deliver DNA into cultured cells. For this purpose, the biotinylated protein was bound to DNA–polylysine complex by a streptavidin bridge [56].

4. Amphipathic peptides with membrane-destabilizing properties

Lytic peptides have important biological functions, mostly in biological warfare. They include defense toxins of insects and fish, antibiotic peptides, or components of the innate immune system of vertebrates (Table 2). Extensive literature exists on these

Table 2
Membrane-active peptides

Source of peptides		Amino acid sequence
Defense toxins		
Melittin	Bee venom	GIGAVLKVLT TGLPALISWI KRKRQQ^{CONH_2}
Bombolitin	Bumblebee venom	IKITTMLAKL GKVLAHV^{CONH_2}
Mastoparan	Wasp venom	INLKALAALA KKIL^{CONH_2}
Crabrolin	Hornet venom	FLPLILRKIV TAL^{CONH_2}
Pardaxin	Fish (shark repellant)	GFFALIPKII SSPLFKTLLS AVGSALSSSGGQE
Antibacterial peptides		
Magainin 2	Frog skin	GLGKFLHSAK KFGKAFVGEI MNS^{CONH_2}
Alamethicin	Fungus	aPaAaAQaVa GLaPVaaEQ
Vertebrate immune system		
Defensins	Granulocytes;	
HNP-1 (human)	disulfide-bridged	ACYCRIPACI AGERRYGTCI YQGRLWAFCC
NP-1 (rabbit)	β-sheet	VVCACRRALC LPRERRAGFC RIRGRIHPLCCRR
Viral fusion proteins (specific for low pH)		
Influenza (X31)	HA2 (N-terminus)	GLFGAIAGFI ENGWEGMIDG WYG–
Influenza (C)	HA2 (N-terminus)	IFGIDDLIIG LLFVAIVEAG IG–
Polio 3	vp1 (N-terminus)	GIEDLISEVA QGALTLVP–
Polio 1	vp1 (N-terminus)	GLGQMLESMI DNTVREVGGA–
Rhino HRV-14	vp1 (N-terminus)	GLGDELEEVI VEKTKQTVAS ISSG–
Rhino HRV-2	vp1 (N-terminus)	NPVENYIDEV LNEVLVVPNI NSSN–
Coxsackie virus	vp1 (N-terminus)	GPVEDAITAA IGRVADTVGT–
Vaccinia virus	14 kD (N-terminus)	MDGTLFPGDD DLAIPATEFF STKA–
Semliki Forest virus	E1 internal sequence	–DYQCKVYTG VYPFMWGGAY CFCD–
Sindbis virus	E1 internal sequence	–DYTCKVFGG VYPFMWGGAQ CFCD–
Viral fusion proteins (active at neutral pH)		
HIV-1	gp41 (N-terminus)	AVGVLGALF LGFLGAAGST MGAASLTLT–
HIV-2	gp41 (N-terminus)	GVFVLGFLGF LATAGSAMGA ASLTLSA–
SIV	gp32 (N-terminus)	GVFVLGFLGF LATAG–
Sendai virus	F1 (N-terminus)	FFGAVIGTIA LGVATSAQIT AGIALAEAR–

a, α-aminoisobutyric acid.

naturally biologically occurring peptides (not listed in this paper). Membrane processes involved in the action of such peptides are discussed in Refs. [57–62]. Table 2 also includes membrane destabilizing element of viral proteins ('fusion domains'). Although the virus-derived membrane-active peptides are not related in sequence, they display some similarities, such as a length of about 15–30 amino acids and an alternating pattern of hydrophobic amino acids interrupted by hydrophilic amino acids. Most of these peptides are able to form amphipathic α-helices. In contrast, defensins represent a family of lytic and fusogenic peptides with β-sheet structure as their active conformation.

Several viral fusion proteins, like the influenza virus hemagglutinin, are activated by the low pH of endosomes. The acidification triggers a conformational change of the protein, extruding the fusion peptide domain from its buried location in the protein. In many cases the fusion peptide is located at the N-terminus of the protein and contains several acidic (glutamic or aspartic acid) residues. Protonation of these residues in the endosome are thought to enhance their membrane-destabilizing activity, although it is unclear whether the latter is induced alone by conformational changes in the peptide (see below). The alternating pattern of acidic and hydrophobic residues is not found in the peptides and fusion sequences with pH-independent activity.

5. Mechanisms for membrane destabilization by amphipathic peptides

For membrane destabilization at least two different mechanisms (and combinations thereof) have to be considered (Fig. 1): rearrangement of lipid packaging (peptide alters membrane properties of the lipid bilayer) or pore formation (self-assembly of peptides in the membrane, without affecting general membrane properties). For a broader review see Refs. [6,7,63,64].

Many peptides destabilize membranes by increasing the negative curvature strain of the lipid bilayer, resulting in a more general disruption of the lipid packaging (see Fig. 1). Local rearrangement from the bilayer phase (L_α) to the hexagonal phase (H_{II}) is facilitated; hypothetic consequences include formation of 'leaky patches'. Membrane-destabilizing class L amphipathic α-helical peptides have a narrow hydrophilic face but a large, space-consuming area in the hydrophobic face of the lipid bilayer. Lysine residues on the hydrophobic phase (allowing deeper insertion into the membrane because of the long methylene side chain) or bulky hydrophobic residues (like tryptophan or isoleucine) cause greater negative curvature strain. Representative examples of this mechanism include the class L peptides magainin, mastoparan, but also other types of peptides like the N-terminus of SIV [65]. In this context it should be mentioned that not all amphipathic peptides are membrane-destabilizing; peptides with so-called class A amphipathic helices [66], like the domains of apolipoprotein A-I, can stabilize membranes, presumably by relieving negative curvature strain (Fig. 1).

In contrast, other lytic peptides self-assemble within the membrane to form a transmembrane pore (Fig. 1), like alamethacin, pardaxin or Bti toxin helix-2 [63]. Consequences of pore formation, like collapse of transmembrane potential and osmotic swelling, lead to lysis of the cell. For this mechanism a 'barrel-stave' model has been suggested: after binding, peptide monomers insert into the lipid bilayer; they aggregate into a barrel-like structure with a central aqueous pore surrounded by peptides; the pore may increase in diameter through progressive recruitment of additional monomeric peptides.

This 'barrel-stave' mechanism differs from the 'carpet-like' mechanism where lipid packaging is disrupted by binding of peptide in almost parallel orientation to the membrane surface, as a consequence of modulation of the negative curvature strain (see above), or charge interaction (positively charged basic residues of peptide with negatively charged phospholipid head groups) and disruption of the hydration shell of the bilayer [59,63].

A particularly well-studied viral fusion peptide is the N-terminus of influenza virus hemagglutinin subunit HA-2. Structural information from X-ray studies at neutral pH [67] and at the pH of fusion [68] suggest that, triggered by the endosomal pH, the fusion peptide is delivered at least 10 nm away from the viral surface, allowing the peptide to interact with membranes. Upon binding to the membrane the peptide adopts an α-helical structure [69–72]. Low

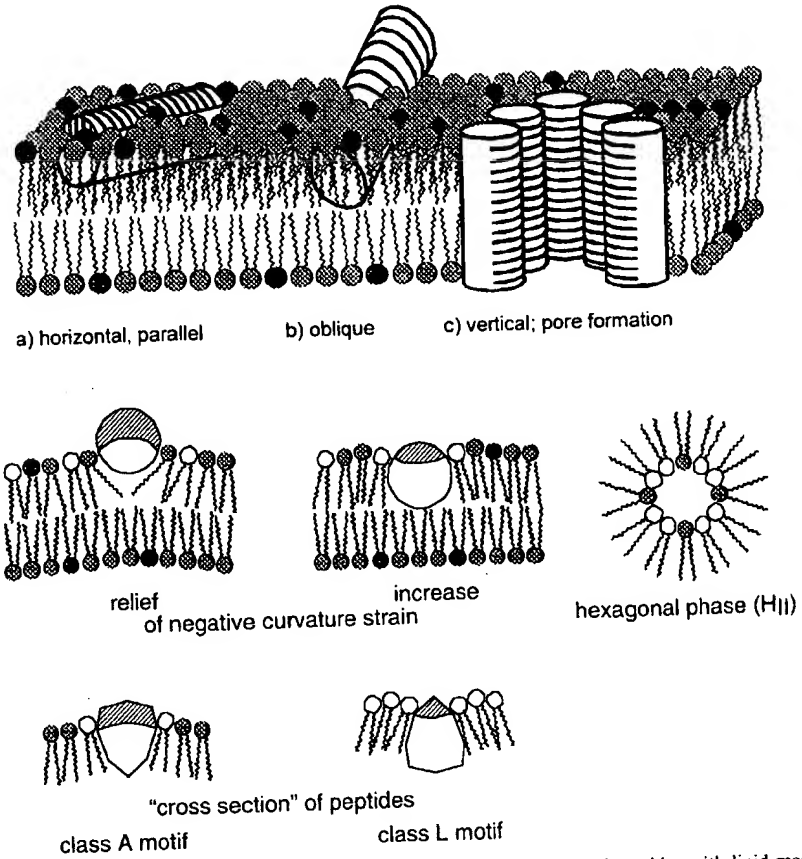

Fig. 1. Mechanisms for membrane destabilization by amphipathic peptides. (A) Interaction of peptides with lipid membrane in horizontal, oblique, or vertical orientation. (B) Peptides with class A motif stabilize membranes by relief of negative intrinsic curvature strain of phospholipid bilayer; class L peptides destabilize membranes by increase of negative curvature strain, facilitating transition to the hexagonal phase.

pH relieves the mutual repulsion of negatively charged acidic residues and seems to promote this transition to an α-helix, but in the presence of the lipid membrane this can also take place at neutral pH. Recent studies show that an oblique insertion of the peptide into the bilayer takes place [73], in a similar way as has been found as a prerequisite of the fusogenicity of the SIV gp32 fusion peptide [65,74,75] or HIV gp41 N-terminus [76].

According to data on recombinant mutant influenza HA proteins and synthetic peptides, the following influenza peptide residues are required for optimum membrane activity. (A) the N-terminal glycine seems to be important for penetration of the N-terminus into the membrane [70,71,77]; substitution of Gly by Ser, His, Leu, Ile, Phe or deletion of Gly resulted in reduction or loss of fusion activity [78]. (B) The residues Trp-14 and Trp-21 play an important role [79]; a peptide lacking Trp-21 (INF3, see Table 3) is almost inactive in the lysis of erythrocytes or cholesterol-containing membrane [11,79]. A peptide with a substitution of Trp-14 by Phe (Table 3, compare INF5 and INF8) is almost 10-fold less active [79]. (C) The influenza sequence contains a conserved stretch of glycines, all located on one side of the helix. Glycines at positions 4 and 8 have been found to be important for activity and/or pH-specificity in the context of the whole hemagglutinin [78]. Replacement of the internal glycines by alanines should also disturb the balance

Table 3
Sequences of synthetic membrane-active peptides

GALA	WEA	ALA	EALA	EALA	EHLA	EALA	EAL	EALA	AGGSC
GALA–GLF	GLFG	ALA	EALA	EALA	EHLA	EALA	EAL	EALA	AGGSC
EGLA-I	GLFL	GLA	EGLA	EGLA	EGLA	EGLA	EGL	EGLA	GGSC
EGLA-II	WEA	GLA	EGLA	EGLA	EGLA	EGLA	EGL	EGLA	GGSC
GALA–INF3	GLF	EAI	EGFI	ENGW	EGLA	EALA	EAL	EALA	AGGSC
HA2 wild-type	GLF	GAI	AGFI	ENGW	EGMI	DGWYG - -			
INF3	GLF	EAI	EGFI	ENGW	EGMI	DGGGC			
INF5	GLF	EAI	EGFI	ENGW	EGnI	DG	K		
	GLF	EAI	EGFI	ENGW	EGnI	DG			
INF6	GLF	GAI	AGFI	ENGW	EGMI	DGWYG			
INF7	GLF	EAI	EGFI	ENGW	EGMI	DGWYG			
INF8	GLF	EAI	EGFI	ENGF	EGMI	DGGG	K		
	GLF	EAI	EGFI	ENGF	EGMI	DGGG			
INF-A	GLF	EAI	EAFI	ENAW	EAMI	DAWYG			
JTS-1	GLF	EAL	LELL	ESLW	ELLL	EAC			
INF9	GLF	ELA	EGLA	ELGA	EGLA	EGWYGC			
INF10	GLF	ELA	EGLA	ELGW	EGLA	EGWYGC			

Peptides INF3, INF5, INF6, INF7, GALA–INF3 and GALA–GLF have been described in Ref. [11]; peptides INF8, INF9, INF10, INF-A and EGLA-I and-II in Ref. [79]; peptide GALA has been described in Refs. [80,81]; peptide JTS-1 in Ref. [82].

of 'helix-breaking' glycines and 'helix forming' residues such as alanines or leucines. In a synthetic peptide (INF-A, see Table 3) the pH specificity was strongly reduced, but the membrane-disruption activity remained high [79].

Membrane fusion and disruption seem to be related processes; the current models suggest the binding and penetration of the fusion peptides into the membrane to be the initial steps. However, leakage and fusion capacities of peptides do not always correlate [83]. It should be emphasized that the characteristics and the activities of fusion peptides may depend on the particular process and environment; for example, it was found that a synthetic N-terminal peptide of HIV-1 gp41 adopts an α-helical structure under conditions supporting pore formation, whereas an antiparallel β-sheet is preferred under conditions promoting fusion [84]. Therefore, beside α-helical conformation also non-helical peptide structures (as in perforins [59]) play important roles in membrane reorganization processes [64,85].

6. Applications for drug and protein delivery

Synthetic membrane-active peptides offer an attractive opportunity to enhance intracellular delivery of drugs by facilitating transmembrane transport. In this context many investigators have focussed on the peptide-mediated endosomal escape of compounds following their endocytic uptake. For this purpose pH-specific peptides can be used which should destabilize specifically endosomal membranes. This means that they must not affect the integrity of the cell surface membrane as do for example the cytotoxic peptides listed in Table 2.

Peptides of virus-derived sequences, mutants thereof, and completely artificial sequences have been investigated. In most circumstances the capacity of synthetic peptides to induce endosomal release by membrane-disruption rather than membrane fusion is required. Therefore, initial activity testing focusses primarily on the capacity of peptides to release marker molecules (like calcein) from liposomes of various lipid compositions, or to lyse erythrocytes. Pioneering studies were initiated in Szoka's group [80,81,86,87]. They designed the acidic amphipathic peptide 'GALA', containing repeats of the motif Glu–Ala–Leu–Ala (one-letter code: EALA), with a high potential for α-helix formation at acidic pH. At neutral pH, the intramolecular repulsions of the negatively charged acidic side chains inhibit the formation of an α-helix (similarly as has been discussed for the influenza virus fusion peptide). GALA has been found to very

efficiently release calcein from phosphatidylcholine liposomes in a strict pH-specific manner. A theoretical model predicts the formation of small leakage pores which allow the release of small compounds like calcein [86] in agreement with observation.

Apart from the results obtained with this amphipathic model peptide our work has also been inspired by the extensive literature published on the membrane-destabilizing activity of peptides analogous to the influenza virus fusion sequence [69–73,77,78]. Tables 3 and 4 show selected examples of amphipathic peptides synthesized and tested in our lab in liposome leakage assays, erythrocyte lysis assays and transfection experiments (see Section 7). Various liposomes were used to evaluate the influence of lipid composition on the membrane disruption [79]. Influenza peptides that differ from the authentic (wild-type) sequence by the introduction of acidic residues (INF5 and INF7) show high activity in leakage of cholesterol-free (PC) and cholesterol-containing (PC/Chol or PC/PE/SM/Chol) liposomes, erythrocytes, and in gene transfer (see Section 7). In agreement with previous work, peptide GALA is very efficient (about approx. 5-fold more than the influenza-based peptides) when cholesterol-free liposomes are used. The activity can be further enhanced by incorporation the N-terminal influenza peptide residues Gly–Leu–Phe (peptide GALA-GLF). However, when using cholesterol-containing liposomes in the leakage assay, optimized influenza peptides (INF5 or INF7) have approx. 100-fold higher efficiency than GALA or GALA-GLF. This is consistent with the reported findings [88] that the interaction of a 14-residue GALA-type peptide with PC monolayers disappeared upon inclusion of cholesterol. The cholesterol effect can be explained by reduced binding of GALA to cholesterol-containing membranes and increased reversibility of surface aggregation of the peptide [89]. Several modifications of influenza-type peptide sequences, such as the replacement of conserved glycines by alanine or substitution of the tryptophans, considerably reduced their activity (see Ref. [79] and below). To further assess the potential role of the glycine array, we compared the GALA sequence with the 'EGLA' sequence (Glu–Gly–Leu–Ala, with one of the alanine residues in the GALA repeat being replaced by glycine). In fact, although EGLA peptides are less active on PC liposomes than GALA, they have considerable leakage activity on Chol-containing membranes (see Table 4).

As a next step, delivery of small compounds (e.g. calcein) or medium-size molecules (e.g. FITC-dextran) into the cytoplasm of cells has been studied. In

Table 4
Efficiencies of membrane-active peptides

Peptide	Leakage activity PC[a]	Leakage activity PC/PE/SM/Chol[a]	Erythrocyte lysis	Transfection efficiency[c]
GALA	30	0.02	<0.003	0.05
GALA-GLF	100	Not tested	0.02	0.03
EGLA-I	1	0.7	0.20	0.1
EGLA-II	1	0.4	0.01	0.1
GALA-INF3	5	Not tested	0.5	0.5
INF3	0.05	Not tested	<0.003	<0.01
INF5	**1**	**1**	**1**	**1**
INF6	0.2 (1[b])	Not tested	2 (0.04[b])	0.006
INF7	0.2 (0.1[b])	Not tested	2	0.7
INF8	0.2	0.15	0.06	0.1
INF-A	0.3 (0.3[b])	1 (0.3[b])	3.5 (0.06[b])	0.1
JTS-1	0.15 (2.5[b])	0.30 (0.3[b])	Not tested	0.03
INF9	0.2	0.15	0.10	0.02
INF10	0.9	0.5	0.4	0.15

Relative efficiencies are given in comparison to peptide INF5 = 1.
[a]Leakage of calcein from phosphatidylcholine (PC), or phosphatidylcholine:phosphatidylethanolamine:cholesterol:sphingomyelin, 10:3:5:2 liposomes (PC/PE/Chol/SM).
[b]Detectable membrane-destabilizing activity at neutral pH: ratio of activities pH 7/pH 5 (when >0.01) is listed.
[c]Relative gene transfer efficiencies in transferrin–polylysine-mediated gene transfer.

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s been studied. In

Transfection efficiency[c]
0.05
0.03
0.1
0.1
0.5
<0.01
1
0.006
0.7
0.1
0.1
0.03
0.02
0.15

sphingomyelin, 10:3:5:2

our investigations we asked whether peptides, when added to the medium of cultured cells, will be internalized and disrupt acidic vesicles instead of being degraded in lysosomes [11,90]. For this purpose cultured cells were incubated with medium containing calcein or 70-kDa fluorescein-labeled dextran (FITC-dextran) with or without influenza peptides. FITC-dextran is taken up into cells by fluid phase endocytosis and accumulates in internal vesicles that appear as bright spots in fluorescence microscopy. When influenza peptides were included, instead of formation of bright vesicles the FITC-dextran had been released into the cytoplasm. Similarly, a peptide-mediated release of internalized calcein also was observed. This effect could be blocked by incubation of cells with bafilomycin A_1, a specific inhibitor of the vacuolar proton pump. This rules out the theoretical possibility that peptides act by forming pores into the cell surface membrane, allowing direct transfer into the cytoplasm. These findings are supported by data obtained when the action of an influenza-type peptide on isolated endosomes was examined. The peptide mediated the release of biotinylated dextran that the living cells had endocytosed prior to endosome isolation in a pH- and sequence-specific manner. The peptide acted on the endosomal membrane at acidic pH from within and from without [32].

Other tests to monitor the transfer across cellular membranes [60,91] utilize an assay usually performed during FACS analysis to mark dead cells. This is based on the exclusion of DNA intercalating compounds (like propidium iodide) from living cells, whereas dead cells or cells permeabilized by the appropriate peptide show increased fluorescence because of intercalation of the dye into the cellular DNA. Using this assay it is difficult to discriminate between cell surface or vesicular permeability; however, pH-specific effects can be monitored by incubating the cells at different pH values.

In addition to the finding that peptides can release labeled macromolecules like dextran, first examples of successful application for the delivery of oligonucleotides [92,93], peptides [94], toxins [95], or genes (see Section 7) have been reported.

An oligonucleotide delivery system described by Liang et al. [92] employs a complex of partially substituted mannosylated polylysine, electrostatically linked to an oligonucleotide. The system utilizes mannose receptor-mediated endocytosis to enhance cellular uptake into alveolar macrophages. Following cellular internalization, the oligonucleotide complex accumulated in endocytic vesicles. Enhanced endosomal exit of the oligonucleotide was achieved using an influenza virus HA-2-based peptide (see above). Improved biological activity of antisense oligonucleotides was also obtained by conjugation to a fusogenic peptide [93].

Partidos et al. [94] have generated a chimeric peptide consisting of a CTL epitope peptide linked to the N-terminal fusogenic sequence of the measles virus F1 protein. Vaccination of mice resulted in the corresponding CTL response, which was not found using a CTL peptide lacking the fusion sequence. The mechanisms by which the fusogenic peptide enhances the CTL response are not known, but it is suggested cell binding, uptake, and translocation through the endoplasmic reticulum of antigen-presenting cells is facilitated. Alternatively, increased hydrophobicity or stability may account for the effect.

As described in Ref. [95] conjugates of transferrin with a chimeric ricin toxin A chain fused to the N-terminus of protein G of the vesicular stomatitis virus showed 10- to 20-fold greater cell killing efficacy than transferrin–ricin conjugates without the membrane-active peptide, suggesting that the ability of VSV protein G to interact with cell membranes can be exploited to facilitate the translocation of a toxin to the cell cytosol.

Besides the delivery of compounds, as described above, approaches for the peptide-enhanced delivery and destabilization of liposomes can be considered as a synthetic alternative to the virosome approach. Work performed in this direction includes Refs. [88,96].

7. Applications for gene delivery

Recent developments of receptor-mediated gene transfer can be considered as important steps toward targeted, synthetic gene delivery systems (selected examples are given in Refs. [97–104]. Complexes of DNA and polylysine-conjugated ligands can be efficiently delivered into cells, but the accumulation

in internal vesicles strongly reduces the efficiency of gene transfer. For instance, asialoglycoprotein or transferrin receptor-mediated delivery into a hepatocyte cell line results in uptake of DNA into practically all cells, but only few cells express the delivered gene [10,26]. In several cell lines and isolates the addition of chloroquine [20] or glycerol [105] considerably increases transfection efficiency, probably by interfering with lysosomal degradation and enhancing the release of the DNA into the cytoplasm. The inclusion of replication-defective adenoviruses or rhinoviruses, either by linkage to the DNA complex or simply by addition to the cell culture medium, has been shown to greatly augment the levels of transferrin-mediated gene transfer (see Section 2 of this review).

Based on these observations and with the intention to combine the advantages of synthetic and viral systems, we generated synthetic virus-like transfection complexes with polylysine as DNA compacting agent, ligands for cell-binding and synthetic endosome-destabilizing peptides as a third important function of viral entry. For this purpose we and others have used synthetic peptides derived from the N-terminus of influenza virus hemagglutinin [10,11,90,106,107] and the rhinovirus VP-1 [31], or artificial amphipathic peptides [11,79,82,108]. Incorporation of these agents has been achieved by chemical linkage to polylysine [106] or positively charged polyamidoamine cascade polymer particles [108], by biotinylation [11] which allows subsequent binding to streptavidin–polylysine, or simply by ionic interaction with polylysine [11,79] or with a polycationic peptide [82]. As described recently [109] a cationic amphipathic peptide KALA (repeats of lysine–alanine–leucine–alanine) was applied to serve for both DNA binding and membrane-destabilization.

In most reported cases gene transfer was strongly ($\geq$10- to $\geq$1000-fold) enhanced by the incorporation of the peptides. Examples of peptides used in the transferrin–polylysine-mediated gene transfer (see Fig. 2) are listed in Table 3. The enhancement of gene expression is strongly dependent on the peptide sequence used (Table 4). Introduction of acidic residues at positions 4 and 7 of the influenza sequence (in addition to the acidic residues at positions 11, 15, and 19) had been shown to enhance the pH specificity of influenza fusion peptides [70,72]. The transfection levels largely (but not completely) correlate with the capacity of peptides to disrupt liposomes of natural lipid composition or erythrocytes in a pH-specific manner (see Table 4). Highest levels of gene expression were obtained with peptides INF5 and INF7. Using peptide INF8 (with the Trp-14 to Phe mutation), transfection levels are

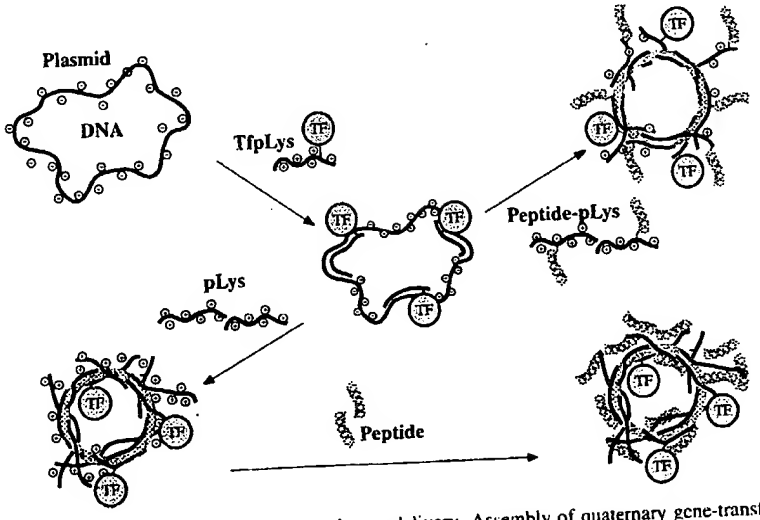

Fig. 2. Synthetic peptide-enhanced transferrin receptor-mediated gene delivery. Assembly of quaternary gene-transfer complexes containing plasmid DNA, transferrin–polylysine, and membrane-active peptides conjugated [106] or ionically bound to polylysine [11].

approx. 10-fold lower, despite incorporation of 3-fold larger amounts of INF8. This correlates with the reduced leakage activity of this peptide. Peptides INF9 and INF10 differ only in residue 14. INF10 (containing Trp-14) has an almost 10-fold higher transfection activity than INF9 (lacking Trp-14).

Peptides with residual activity at neutral pH (INF6, INF-A, JTS-1) were found to induce considerable toxic side effects when used in transfection. Although not critical in the context of the whole influenza virus hemagglutinin, where the N-terminus is buried at neutral pH, the pH specificity apparently can be essential in the context of the peptide-based delivery system, where cytopathic effects due to the disruption of the plasma membrane must be avoided. Additionally, it has to be emphasized that the efficacy of different peptide sequences in gene delivery also largely depends on the exact formulation of the DNA complex and on the cell type to be transfected. This explains for example, why peptide JTS-1 has been found to be most effective in a cationic peptide-mediated DNA delivery system lacking a cell-binding ligand [82], whereas the peptide had low activity in transferrin–polylysine-mediated delivery (see Table 4 and below).

Amphipathic peptides also have been used to assist in lipid-based gene delivery. The cationic, amphipathic peptide gramicidin S when included into a DOPE lipid/DNA composition, has been shown to strongly facilitate DNA delivery [110,111]. Incorporation of gramicidin S and DNA into asialofetuin-labeled liposomes was used for receptor-mediated gene transfer into primary hepatocytes [112]. The influence of influenza-based peptides on cationic lipid-based transfection has been investigated by us [113] and others [114,115]. When positively charged lipospermine/DNA complexes were used, only a slight but significant (up to 5-fold) enhancement of the gene expression was obtained by association with the peptides. Thus, endosomal escape seems to be no major barrier for optimized, positively charged DNA/lipospermine complexes. However, it can be a bottleneck for less-charged complexes; here gene transfer efficiency is increased by a factor of 50–1000 by peptides INF6 (influenza virus-derived sequence) and INF10 (artificial sequence, see Table 3). Interestingly, peptide INF6 (not pH-specific!) was the most active peptide in this setting, whereas it displayed low efficiency and toxicity in the polylysine-based system.

8. Conclusions and prospects

First reports have been published on the application of membrane-active peptides for intracellular delivery of small compounds like calcein, oligonucleotides, peptides, or macromolecules like plasmid DNA. However, in no case did the efficiencies of intracellular delivery come close to the efficiencies observed with the naturally evolved translocation mechanisms of viruses or protein toxins.

Our knowledge of the action of membrane-destabilizing peptides is still very limited. Although the primary sequences of numerous natural membrane-active peptides and protein domains are known, the role of secondary structure and the role and mechanism of assembly into larger aggregates in the context of interaction with membranes are less well understood. The experimental tools for assessing peptide activity used so far (i.e. leakage and fusion assays, with artificial or natural sources of membranes) provide us only with very crude information. More sophisticated experimental techniques and a considerable amount of research work will be required to put the development of peptide sequences assisting in drug delivery on a less empirical basis.

Besides the 'quality' of the peptide linkage, the 'quantity' of the peptide load may be very relevant; The other major challenge lies in the construction of the delivery vehicle. The way of incorporation of peptides into the delivery vehicle may strongly influence their activity. Because of the multimolecular nature of membrane-destabilization, a critical concentration of active peptides is required within the delivery particle. For this purpose also concepts for noncovalent loading have to be considered. Novel ways of inhibiting the peptides' activity outside the cell and of triggering their release into the active conformation within vesicles will have to be found. A better understanding of the secondary/tertiary structure–function relationship will help us to exploit alternative cellular mechanisms and novel coupling strategies for releasing the peptides into their active modes. For example, the assembly of peptides into the active multimeric forms may be

inhibited by coupling them to carriers via a linkage that will be specifically cleaved during the uptake or by sequence-specific proteases within endosomes or lysosomes.

In this respect, progress both in the molecular understanding of the peptide–peptide and peptide–lipid membrane interactions, and the development of improved carriers as well as an even better definition of biological uptake and transport processes will be pace-making for the generation of efficient synthetic peptide-based delivery system.

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